Designing and Characterization of Emulsion-Based Matrices for the Encapsulation of Bioactive Oils using Polysaccharides

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by

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Prof. Preetam Sarkar

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This is to certify that the work presented in the dissertation entitled *Designing and Characterization of Emulsion-Based Matrices for the Encapsulation of Bioactive Oils using Polysaccharides* by *Lohith Kumar DH*, Roll Number 614FT1004, is a record of original research carried out by him under my supervision and guidance in partial fulfillment of the requirements of the degree of *Master of Technology (Research)* in *Food Process Engineering*. Neither this dissertation nor any part of it has been submitted earlier for any degree or diploma to any institute or university in India or abroad.

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In Loving Memory of My Parents and Lord Hanuman

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Declaration of Originality

I, *Lohith Kumar DH,* Roll Number *614FT1004* hereby declare that this dissertation entitled *Designing and Characterization of Emulsion-Based Matrices for the Encapsulation of Bioactive Oils using Polysaccharides* presents my original work carried out as a master student of NIT Rourkela and, to the best of my knowledge, contains no material previously published or written by another person, nor any material presented by me for the award of any degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the sections Reference". I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

I am fully aware that in case of any non-compliance detected in future, the Senate of NIT Rourkela may withdraw the degree awarded to me on the basis of the present dissertation.

January 18, 2017

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Abstract

Flaxseed oil has abundant polyunsaturated fatty acids that are important to health but are oxidized easily during storage. These bioactive lipids are susceptible to chemical deterioration causing nutrient loss and development of undesirable off-flavors. Oil-in-water emulsions could be used as an effective tool to protect these functional active lipids from degradation by engineering the emulsion droplet interfacial layer. Engineering oil/water interfaces of emulsions has been studied extensively, but practical technologies are still demanded by the food industry. Emulsions are a category of encapsulation systems applicable to various bioactive compounds in food formulations. Emulsions have a fluidic oil phase which facilitates the mobility and release of bioactive compounds. Emulsion based encapsulation systems has been exhibited to have potential applications in functional food formulation. They have been used to inhibit lipid oxidation, triggered or controlled release during different processing and storage conditions. Biopolymer characteristics (e.g., molecular weight, charge density, and conformation) and droplets characteristics (e.g., size, charge, and concentration) are most significant aspects in the formulation of stable emulsions.

In this study natural biopolymer pectin and chitosan has been used to produce oil-in-water emulsions. In first part of study, primary emulsion was produced from pectin and influence of sonication time, pH, NaCl, surfactant to oil ratio was studied. It was found that pectin emulsion at a pH primarily had less charge density and then changing the pH conditions to where they had more charge density (4.0 to 8.0) led to the development of less stable primary emulsion. The pectin layered droplets in the primary emulsions were stable to creaming and droplet aggregation at temperatures at 100 ^{o}C and NaCl concentrations up to 120 mM. However, droplets were more prone to instability due to coalescence at less pectin concentration.

In the next part of the thesis, bi-layered emulsions were created using layer-by-layer approach. Pectin was used to stabilize a primary emulsion with less droplet size,

then, a cationic chitosan biopolymer was coated to the primary emulsion to produce secondary emulsions with cationic charge around the droplet. The interface of pectin and chitosan as a function of pH, NaCl and biopolymer concentration was studied with the anticipation of drawing conclusion for their interaction at droplets surfaces. Chitosan and pectin interaction to form insoluble or soluble polyelectrolyte complex at the droplet interface was dependent on the pH. At pH conditions where chitosan and pectin have opposite droplet charges (pH 3.5, 4.0 and 5.0) they interact intensely to form polyelectrolyte complexes. Stable and tailored pectin-chitosan secondary emulsions however could be formulated. But, confined range of chitosan concentration (between depletion concentrations and saturation) and protonation of chitosan are important factors in preparation of pectin-chitosan bi-layered emulsion.

The objective of this research was therefore to understand how emulsion interface properties and interactions among emulsion droplets influence lipid oxidation in food emulsions. In addition, this study delivers valuable evidence on practice of the layer-by-layer approach for production of bi-layered food grade emulsions with an emphasis on the effects of formulation conditions and biopolymer properties on long term emulsion stability.

Keywords*: *Flaxseed oil*; *Pectin*; *Chitosan*; *Encapsulation*; *Oil-in-water emulsion*; *Bi-layer emulsion.

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List of Abbreviations and Symbols

Abbreviations

BHA - Butylated Hydroxy Anisole
BHT - Butylated Hydroxy Toluene
BSA - Bovine Serum Albumin
DA - Degree of Acetylation
DD - Degree of Dissociation
DE - Degree of Esterification
DGR - Droplet Growth Ratio
DLS - Dynamic Light Scattering
ELS - Electrophoretic Light Scattering
ESI - Emulsion Size Index
GRAS - Generally Recognized as Safe
HMP - High Methoxyl Pectin
LAE - Lauric Arginate
LMP - Low Methoxyl Pectin
MDD - Mean Droplet Diameter
OSA - Octenyl Succinic Anhydrides
TBARS - Thiobarbituric Acid Reactive Substances
TBHQ - Tert-Butyl-Hydroxy-Quinone
UV - Ultra-Violet

Symbols

τ - Shear stress (**Pa**)
γ - Shear rate ($\mathbf{s^{-1}}$)
λ - Wavelength
ω-6 - Family of polyunsaturated fatty acids ($C_{18:2}$)
ω-3 - Family of polyunsaturated fatty acids ($C_{18:3}$)
K - Consistency Index ($\mathbf{Pa.s^n}$)
n - Flow Behavior Index
$\mathbf{A_{400}}$ - Absorbance at 400 nm
$\mathbf{A_{800}}$ - Absorbance at 800 nm
$\mathbf{D_0}$ - Mean Droplet diameter of fresh emulsion (**nm**)
$\mathbf{D_{14}}$ - Mean Droplet diameter of emulsion after fourteen days of storage (**nm**)
$\mathbf{D_7}$ - Mean Droplet diameter of emulsion after seven days of storage (**nm**)

Chapter 1

Introduction

1.1 Background

Practically all foods products deteriorate at some rate. The rate of deterioration is dependent on the food formulation, composition, structure, packaging and storage regime. This deterioration can be termed as "quality loss". Food quality is an integrated measure of food texture, flavor, color, purity and appearance (Lett et al. 2016). Fertilization, sustainable agriculture practices, irrigation, pest control, harvesting site sanitation, personnel hygiene, transportation, processing, and storage steps determines the final quality of food. Nevertheless, an interaction between food ingredients and surrounding environment also leads to "quality loss" of food. For example, ***Maillard browning, enzymatic browning, and lipid oxidation*** are important quality deteriorating processes encountered in food production**.** Maillard browning is a non-enzymatic reaction between monosaccharides carbonyl and α-amino groups in food which gives flavor and color during frying, roasting and baking (de Lavergne et al. 2016). Enzymatic browning is the rapid, polyphenol-oxidase mediated conversion of phenolic compounds into dark polyphenolic polymers. In order to inactivate enzymes, food items must be blanched or pretreated prior to processing. Bioactive lipids (ω-3, ω -6, butyric acid, triglycerides, and long chain fatty acids) are major part of functional foods in recent years. Due to their susceptibility to oxidation their utilization is limited (Salminen et al. 2016). To control lipid oxidation in food systems, many antioxidants are used. However, though they fall under generally recognized as safe (GRAS) list, long term consumption lead to health issues related to aging and age-related diseases due to modification in lipid profile in the body (Yoshihara et al. 2010).

Technological advances have made possible better food processing operations since important aspects such as the elimination of toxins, prevention of pathogens, preservation, and improved consistency can be accounted for in more detail, generating processed foods that are less susceptible to decay than fresh foods, an important point for transportation and storage. All these aspects of food processing are more effectively achieved by the incorporation of functional compounds through encapsulation.

Encapsulation systems play an important role in the food processing sector, maintaining functional properties of active compounds through encapsulating in colloids, emulsions and other biopolymers matrices.

In many food matrices lipids are present in the form of dispersed oil droplets in a continuous aqueous phase. This aqueous phase is capable of carrying several water-soluble species such as transition metal (Frankel 2014). High reactivity of iron ions in aqueous matrix makes it potent pro-oxidant at high concentration. Lipid peroxides are broken down into reactive radicals more profoundly in the presence of ferrous ion through Fenton reaction mechanism (Frankel 2014; Chaiyasit et al. 2007). Ferric ion can also participate in similar reaction giving rise to radicals; however the rate of reaction is ten times slower than that of ferrous ion. Apart from presence of transition metals, reducing agents such as singlet oxygen, ascorbic acid and compounds with thiol groups accelerates iron induced lipid oxidation through the reduction of ferric ion to ferrous ion. In emulsions, containing negatively charged oil droplets, transition metal ions are attracted to the interface, thus accelerating the oxidation process than that of positively charged oil droplets. This behavior is due to the electrostatic attraction between positively charged transition metal ions and negatively charged oil droplets resulting in increased formation of free radicals at the interface (Mei et al. 1999). Such generalization has been made based on the observations of accelerated lipid oxidation and principles of charge interactions.

Protection of bioactive lipids especially poly-unsaturated fatty acids is the need of the hour. In this context, encapsulation of bioactive lipids in colloidal matrix is the most successful and widely used process to protect bioactive lipids from oxidation in food industry (Rodríguez et al. 2016). Food colloids are multiphase matrices comprising bioactive compounds or other structures with characteristic spatial dimensions in the colloidal size rage such as emulsions, gels, and foams. Oil-in-water emulsions are one of the most important classes of food colloids and include food products such as milk, cream, dressing, mayonnaise, soups, sauces and beverages. Generally, food colloids are complex structures because of the molecular properties and interactions of the three principle constituents (water, oil and emulsifier) (Aboalnaja et al. 2016). Aspects influencing emulsion characteristics include interaction of components, emulsifier-co-emulsifier interaction and emulsifier-droplet interactions, lipid oxidation at the interface and process condition for instance pressure, temperature, time and mechanical agitation. It can thus be relatively challenging to formulate stable emulsions. Ultimately, to produce a structured

designer emulsion, one needs to understand the fundamental aspects of interfacial engineering and properties of emulsifiers and its relation with droplet interfaces. Food grade biopolymers such as proteins and polysaccharides are used as interfacial stabilizing materials extensively (Ozturk et al. 2016). However, a single biopolymer may not be sufficient to achieve all functional properties of emulsion based encapsulation system. In this perspective, researchers are utilizing the polyelectrolyte behavior of food biopolymers to generate novel bi-layered emulsion matrices. Yet, these are limited to protein-polysaccharide combination. In this study, we explored the utilization of *polysaccharide-polysaccharide* polyelectrolyte behavior to generate novel interfacial stabilization thereby protecting bioactive lipid rich flaxseed oil from oxidation reaction.

1.2 Motivation

The motivation for the work contained in this thesis is twofold: (1) primary polysaccharide emulsion formation and characterization (2) structuring of primary polysaccharide emulsion through interfacial engineering and layer-by-layer electrostatic deposition concepts. Colloids are essential structures in food processing. Utilization of their functional properties of encapsulation led many discoveries. In the fundamental realm, *David Julian McClements* and his coworkers conducted numerous pioneering experimental studies for the factors that influence the lipid oxidation in emulsions. In this perspective, emulsions as the encapsulation matrices can be developed for oils having bioactive properties (McClements 2010). Lipid oxidation, a major concern in food production and processing is often a determining factor in the quality, shelf-life, and safety of foods. Investigations have been extensively conducted to study lipid oxidation in bulk oils and fats (Chaiyasit et al. 2007). However, lipids exist as dispersed systems, such as emulsions in most processed foods. In such cases, oxidation of dispersed oils is influenced by many factors greatly different from those in the bulk oils and fats. The factors that influence lipid oxidation in dispersed systems are still poorly understood. To effectively manage lipid oxidation in emulsions, it is imperative to design a novel interfacial layer.

1.3 Problem Statement

Lipid oxidation is the one of the main causes of chemical instability of food lipids resulting in quality deterioration of many food products. Dietary lipids are mainly consumed not as bulk oils but in an emulsion form. Although many factors influence the

rate of lipid oxidation in emulsion systems, the nature of the interfacial membrane of an emulsion droplet has a large impact on the rate of lipid oxidation probably due to the interactions of the lipid hydroperoxides located at the droplet surface and transition metals from the aqueous phase. It is possible to retard oxidative degradation of emulsified oils by careful selection of emulsifiers and by systematically controlling the characteristics of the interfacial layers surrounding the oil droplets (e.g. thickness, charge, rheology, permeability and chemical reactivity) (Kiokias et al. 2016). One such approach is layer-by-layer electrostatic deposition. One of the principal difficulties stated in studies that have used the layer-by-layer electrostatic deposition approach to produce multilayer emulsions is extensive flocculation of the droplets (Guzey et al. 2006). Flocculation can be delayed by vigilantly modulating the emulsion preparation conditions, for instance, biopolymer characteristics (e.g., as molecular weight, charge density, and conformation) and colloidal particles characteristics (e.g., size, charge, and concentration), and the characteristics of the solvent (e.g. as pH, dielectric constant, and ionic strength) (McClements 2010). The choice of biopolymers is one of the most important factors in the production of oil-in-water emulsions using the layer-by-layer electrostatic deposition technique. If these emulsions are considered for use in food products they have to be prepared from natural biopolymers. Most food biopolymers are complex, non-homogeneous materials. Their specific properties depend on their source, isolation and purification (Dickinson 2003; Charoen et al. 2011). Very often they are not well characterized and they contain impurities. These factors make food emulsions as challenging matrices to produce using the principles of layer-by-layer deposition. It would be highly useful to have fundamental understanding of by what means these factors affect the development of stable bi-layer emulsion.

1.4 Specific Objectives

The objective of this research was to evaluate lipid oxidation in polysaccharide emulsions. Through this research lipid oxidation in polysaccharide emulsions will be better understood, thus potentially providing information on new combination which could be used to stabilize dispersed food lipids. The specific objectives are

1. *Formulation of Pectin Stabilized Oil-in-water Primary Emulsion*; Investigation of effect of sonication time, pectin concentration, sodium chloride

concentration, pH and environmental stress factors on emulsion properties and oxidative stability of flaxseed oil-in-water emulsion.

2. *Formation of Pectin, Chitosan bi-layer Oil-in-water Emulsion*; Investigation of effect of chitosan concentration and environmental stress factors (pH, sodium chloride concentration and thermal treatment) on bi-layer flaxseed oil-in-water emulsion properties and oxidative stability.

1.5 Thesis organization

The thesis is divided into five chapters organized as follows:

Chapter 1: Introduction

This chapter describes background, motivation, thesis problem of statement and objectives.

Chapter 2: Review of Literature

Chapter 2 presents a brief review of lipid oxidation in emulsions, different emulsifiers, formation of emulsion with a focus on fundamental aspects.

Chapter 3: Material and Methods

The chapter elaborates on the materials and experimental techniques used in this thesis.

Chapter 4: Results and Discussion

4.1. Pectin Stabilized Emulsion; Influence of Sonication Time, Pectin Concentration, Sodium chloride and pH

This chapter focused on pectin layered primary emulsion. Role of sonication time and emulsion composition on oxidative stability is discussed.

4.2. Structured Pectin, Chitosan Bi-Layer Emulsion: Protection of Flaxseed Oil against Lipid Oxidation

This chapter provides the insight into polysaccharide based bi-layer emulsion properties and ability to inhibit lipid oxidation in emulsion matrix.

Chapter 5: Conclusions

Chapter 5 concludes the results presented in the results and discussion section.

Chapter 2

Review of Literature

The word emulsion originates from the Latin word ‘emulgere’ which means ‘to milk’. An emulsion, such as milk, is a metastable colloidal mixture of two immiscible liquids (Lissant 1988). In such colloidal mixture, one of the liquids is distributed as tiny droplets into the other. These droplets are stabilized by amphiphilic molecules at the interface. The diameter of an emulsion droplet can range from 0.01-100 μm (McClements 2015). Droplet size of emulsion determines many important characteristics of food such as appearance, texture, shelf-life, and flavor. Therefore it is imperative to measure, control and report droplet size in emulsion system (McClements 2002). Emulsions are utilized in many fields, including pharmaceuticals, foods, cosmetics and petroleum. In addition, emulsions are often used as model systems to understand the behavior of more complex food systems since the science of emulsions has reached a stage at which a deliberate manipulation of colloidal properties has become feasible (McClements 2010). The results gained from these carefully chosen experiments can be applied to a large variety of more complex “real-life” application. This will ultimately help enhance food quality and prolong shelf life.

Emulsion system containing oil droplets dispersed in aqueous phase are oil-in-water emulsions, while, water droplets dispersed in oil phase are referred as water-in-oil emulsions. In food systems, both oil-in-water (e.g., soups, milk, salad dressing, mayonnaise, beverage, creams) and water-in-oil (e.g., butter, margarine, spreads) emulsions have gained considerable attention for encapsulation of functional molecules (McClements 2015). Besides, emulsions can have one internal phase or emulsions can have internal phase that are themselves in an emulsified state. These emulsions are called multiple or polyphasic emulsions. An example of such emulsion is a water-in-oil-in-water double emulsion. The internal phase of such emulsion consists of water-in oil-emulsion that is dispersed in water. This type of emulsion is mainly used to produce fat reduced margarine and spreads (Kiokias et al. 2016).

A typical oil-in-water emulsion has three components: (1) the internal oil phase, (2) the continuous phase in which the oil droplets are distributed and (3) the interfacial region made up of amphiphilic molecules referred to as emulsifier. Emulsifiers in the emulsions align themselves to the oil-water interface to form an interfacial layer depending on their polarity and concentration (González et al. 2015). Food emulsions may consist of various constituents including lipids, proteins, polysaccharides, minerals, water, and sugars. These constituents reside in the individual phases and structural components by means of covalent and physical interactions, providing the end product with its characteristic physicochemical properties (McClements et al. 2009). In general, non-polar entities are positioned in the lipid phase, polar entities in the aqueous phase, and amphiphilic entities at the interface (Ong et al. 2015). However, at equilibrium, molecules continuously change locations within the emulsion, and molecules can also move if there is a change in temperature or in response to other environmental stress factors (e.g., ionic strength, salt and pH) (Aoki et al. 2005).

Much advancement has been achieved in the field of encapsulation systems for enhancing the bioavailability. Encapsulation systems such as emulsion have a long-standing history of being utilized as a protective mechanism for many active ingredients (Gibbs 1999). This is because the effectiveness of nutraceuticals to provide therapeutic or physiological benefits greatly depends on the bioavailability of key active ingredients to the target site of action. Factors such as poor aqueous solubility, insufficient residence time in the gastro intestinal tract, instability to changing physiological environments, low transport coefficient across the intestinal lining and susceptibility to rapid metabolic transformation could significantly lower the efficacy of nutraceuticals in disease prevention (McClements et al. 2009; Ahmed et al. 2012). Among all systems, emulsion is one of the most exploited and convenient encapsulation approaches to protect the active components having low bioavailability. Depending on formulation composition and processing methods, different types of emulsion systems can be conveniently customized to fit specific delivery needs. Specifically, oil-in-water emulsion is demonstrated to be exceptionally effective to enhance solubility, absorption, transportation, and bio-efficacy of lipophilic functional food ingredients using *in vitro* and *in vivo* models (Park et al. 2007; McClements et al. 2010).

2.1 Emulsion Classification

A generalized classification of emulsions based on the size of the dispersed phase globules are given as follows.

2.1.1 Conventional emulsion

Conventional emulsions have droplet size in the range of 10 to 100 μm. Solubilized lipids are mainly at droplet interior. They are thermodynamically stable. Conventional emulsion, also known as macroemulsion, is composed of two liquids whose density varies considerably (McClements 2015). They are distributed as droplets in a continuous phase by action of mechanical shear. The droplets in the dispersed phase typically have mean droplet diameter within the range of 10 – 100 μm. Since the size of disperse phase droplets falls into the same order of light wavelength ($d \approx \lambda$), conventional emulsions typically appear as cloudy or opaque liquid systems, with the highest light scattering value at $d \approx 200$ nm (McClements et al. 1998). Furthermore, due to its relatively large droplet size and thermodynamically unstable nature, conventional emulsion is prone to gravitational separation and droplet aggregation, which eventually leads to phase separation upon storage. The viscosity of emulsion systems can be altered through selection of different emulsifiers, the size and concentration of dispersed droplets, and the presence of foreign particles (Dickinson 2003; Matos et al. 2015). Nevertheless, since the formation of conventional emulsion usually involves lower energy input and simpler processing requirements compared with emulsions having a much smaller droplet size, conventional emulsion is still currently the most commonly utilized form of emulsion in the food industry (McClements 2010).

2.1.2 Microemulsion and Nanoemulsion

In general, microemulsion has a droplet diameter ranging between 0.2 – 5 μm, while droplet diameter of nanoemulsion is about 20 – 100 nm. Solubilized lipids are mainly at interfaces in case of nanoemulsions. These systems are thermodynamically unstable and optically transparent because the droplets of oil or water are very small (Karadag et al. 2013). Micro- and nanoemulsions, sometimes together referred to as submicron emulsions, are systems with much smaller dispersed droplets than conventional emulsion (McClements 2012). The formation of microemulsion occurs spontaneously over time and requires no energy input for particular composition ratio of dispersed phase,

continuous phase, and emulsifier. The spontaneously-formed microemulsion is resistant to any types of phase separation, even for prolonged storage periods, as it is thermodynamically stable at the lowest free energy state (McClements 2012). The nanoemulsion system, on the other hand, is a metastable but thermodynamically unstable system that will eventually phase separate upon long-term storage (Aboalnaja et al. 2016; Leong et al. 2009). Yet, due to the small mean droplet diameter (MDD), nanoemulsion is sustained quite well on separation caused by gravitational force and, thus, can remain kinetically stable for a relatively longer period than conventional emulsion. Moreover, the attractive force between dispersed droplets is smaller with the smaller MDD and aggregation is then reduced (McClements 2012). Both micro- and nanoemulsion systems appear as transparent liquid solutions, since MDD is much smaller than the wavelength of light ($d << \lambda$) and scattering of light is minimum (Chantrapornchai et al. 1998). The rheological property of submicron emulsion can also be altered through selection of formulation materials and processing conditions. However, when the concentration of the dispersed phase is the same for both conventional and nanoemulsion systems, nanoemulsion may have a slightly higher viscosity, since the hydrodynamic interaction between droplets increases as the distance between droplets decreases due to denser packing of smaller droplet unities (Tadros 2015). Nanoemulsion with various favorable characteristics has become an emerging topic for advanced encapsulation systems. The small size, stability, and the transparent property of submicron emulsion offer many exciting possibilities in nutraceutical delivery and development of novel functional foods (Ahmed et al. 2012; McClements et al. 2007).

2.2 Emulsion Formation

Emulsion systems are innately thermodynamically unstable. Hence, the energy required to create emulsions involves generating a large increase in the interfacial area between the two immiscible phases (Canselier et al. 2002). Mixers and blenders can be used to form emulsions, but a problem with such instruments is formation of undesirable foam at high speed. Sonicators, colloid mills, and valve homogenizers are used both in the laboratory and in industry for the preparation of emulsions (Abbas et al. 2013). In sonication, the absorption of the ultrasonic wave takes place within a few centimeters of the emitter. The high power ultrasound is proliferated via successions of rarefaction and compression which cause quick generation and collapse of cavities in the solution. There

is a lot of energy released when a cavity collapses. The developed cavity bubbles referred as localized 'hotspot' produces temperature of near 4000 K and pressure more than 100 MPa during less than 0.1 μs (Abbas et al. 2013; Ashokkumar 2015).

High pressure homogenizer is an equipment used for emulsification. The principle of the valve homogenizer involves pumping the liquid mixture through a narrow orifice (0.1 mm) under high pressure (5-40 MPa or higher). Homogenization action results in shearing forces developed by rapid motion (50-200 ms^{-1}) of the liquids through the orifice (Schultz et al. 2004). Only a very small fraction (<<0.1 %) of the applied energy is actually stored in the emulsions in the form of interfacial free energy with the rest of the energy being dissipated as heat. The average residence time of pre-mixed emulsions in the homogenizer valve is less than a second. The applied pressure causes drop of vapor pressure of the liquid resulting in development of unstable steam bubbles. The collapse of these steam bubbles releases the energy to disrupt oil droplets (Yuan et al. 2008). Hence, the cavitational steam bubbles provide the major contribution to the droplet disruption. The efficiency of the oil droplet disruption and formation of uniform droplet size in a pressure driven homogenizer is directly related to the pressure difference in the homogenization system.

2.3 Food grade Interfacial Stabilizing Molecules

Emulsifiers that can be used to form emulsions include charged polyelectrolytes like protein, ionic surfactants and polysaccharides. It is necessary that these hydrocolloids interact at the oil/water interface and create a droplet with a certain electric charge. Every emulsifier has its unique property such as the characteristics of emulsifiers could be either beneficial or detrimental in a particular application (Solans et al. 2012; McClements et al. 2010). Therefore the choice of emulsifier finds great significance. However, natural biopolymers can be considered as the main inventory for suitable materials to be used as interface stabilizing molecules in emulsion formation. Polysaccharides and proteins are two categories of natural biopolymers that can be used in emulsion based encapsulation and each category has its advantages and limitations (Table 2.1) (Charcosset 2016).

Table 2.1: Advantages and limitations of polysaccharides and proteins as nutrient carriers for bioactive food ingredients.

Polysaccharides	Proteins
• They are safe, biocompatible, biodegradable	• They are safe, biocompatible, biodegradable
• Can be modified to achieve the required properties	• Have a wide range of functional properties
• Versatile carriers to bind and entrap a variety of hydrophilic and hydrophobic bioactive food ingredients	• Consistent composition and properties
• They are considered as a suitable shell under high temperature processes	• Can interact with wide range of hydrophilic and hydrophobic bioactive compounds
• Resistant to gastric and intestinal conditions	• Can be easily restructured in nano forms by several physical and chemical treatments
• Slightly affected by pH and ionic strength of the solution	• Liberates on enzymatic digestion
• Their composition and properties are greatly affected by the source and method of extraction	• Easily affected by change in pH and ionic strength
	• Poor resistance to intestinal conditions
	• May develop allergic reactions

2.3.1 Proteins

Proteins can be obtained from plant, animal or marine sources. The most prominent source of proteins used as emulsifiers include the milk proteins such as whey proteins and casein. Milk proteins are relatively abundant in availability and can be isolated with ease. Caseins are major milk proteins constituting up to 80% of total milk proteins. With respect to structure, proteins are highly disordered and relatively flexible proteins. They are known to compose of the four structural fractions- αS_1-casein; αS_2-casein; β-casein; and κ-casein. The next major milk protein fraction is whey, making up to 20% of the total milk protein. They in turn consist of several fractions including α-lactalbumin, lactoferrin, β-lactoglobulin and various other minor components (Hu et al. 2003). The emulsifying actions of these proteins are attributed to the amphiphilic nature which enables them to simultaneously interact to the oil and water phase, forming a bridge. They prevent the droplets from coming closer and forming aggregates by steric and electrostatic repulsion mechanisms. The electric charge on the protein molecules is

conferred by size, location, number of charged groups such as ionisable amino acids and other phosphates present on the primary polypeptide background. Every protein has its characteristic isoelectric point (pI). It is the pH at which the total number of positive groups and the total number of negative groups on the protein is equal giving a net zero charge. At pH values above isoelectric point, proteins will possess a net negative charge in solution and at pH values below isoelectric point, the net charge on the protein is positive (McClements 2004).

When proteins stabilize emulsion interfaces, they tend to form thin interfacial membrane around fat globules. Electrostatic repulsion between the droplets is the major mechanism that keeps the droplets in dispersed form. Also, these systems are sensitive to the aqueous phase conditions. For example, emulsion tends to destabilize near protein isoelectric pH. A commonly used protein that forms thin interfacial layer is β-lactoglobulin. The emulsions using β-lactoglobulin may destabilize in response to changes in pH and salt concentration. By making the interface thick, the emulsion droplets receive stabilization by a combination of steric and electrostatic mechanisms and thus become stable to change in pH and ionic strength (Mao et al. 2012a; Mao et al. 2012b). This is supported by the observation that lactoferrin emulsified lipid globules are stable to pH and ionic strength, when the interface is coated with sufficient emulsifier molecules. This ability of lactoferrin is due to the formation of thick interfacial layer attributed to its high molecular weight and to the carbohydrate side chains that protrude into the water phase. If surface coverage is poor, aggregation of fat droplets occurs (Tokle et al. 2011). Also, counter-ions in solution happen to influence lactoferrin covered oil droplets. Multivalent anions of phosphate and acetate buffers bring down the charge conferred by lactoferrin, thus changing the physicochemical behavior of the emulsion system (Schmelz et al. 2011). Functional performance is determined by the temperature sensitivity of globular protein. Above thermal denaturation temperature, globular protein unfolds, inducing conformational changes in proteins like bovine serum albumin (BSA), lactoferrin and β-lactoglobulin. These changes expose amino acid groups such as sulfhydryl groups or carboxylic groups leading to interaction among proteins themselves. When these interacting proteins are present in different fat droplets the emulsion structure may collapse due to protein-protein hydrophobic interactions and induction of di-sulphide linkages (McClements 2004). On the contrary, when caseins are heated, temperature

induced conformational changes are not noticeable. Therefore, caseins exhibit better stability during thermal treatment (Hu et al. 2003).

2.3.2 Polysaccharides

Several natural and modified carbohydrates can act as emulsifiers provided they exhibit amphiphilic nature. For example, gum Arabic has polar and non-polar groups naturally present while modified starch is made amphiphilic by linking the hydrophilic starch to hydrophobic octenyl succinic anhydrides (OSA) through covalent linkages, thus creating a single entity (Nakauma et al. 2008). The OSA groups align themselves on the oil surface while the starch part of modified starch protrudes into the aqueous phase (McClements et al. 2007). Several studies indicate that modified starch forms an emulsion with overall negative charge that is stable over a wide range of pH (2.0-9.0). Similarly, gum Arabic naturally has a small protein fraction making it a natural protein-polysaccharide conjugate (Charoen et al. 2011). Though the protein fraction is small, it helps the adherence of the emulsifier molecule on the oil side of oil/water interface, with the polysaccharide part in water. Gum Arabic also forms anionic emulsion droplets. Both gum Arabic and modified starch stabilize emulsions by combining the mechanisms of steric hindrance and electrostatic repulsion (McNamee et al. 1998). In general, polysaccharide stabilized emulsion tend to be more stable to stress induced by pH, ionic concentration and temperature (Chanamai et al. 2002). In this study, chitosan and pectin polysaccharides are used to stabilize the flaxseed oil-water interfaces and details of these biopolymers are discussed below.

Chitosan

Chitin is one of the most abundant natural polymers and its major industrial sources are crab, lobster, or shrimp shells. Chitin corresponds to an insoluble material in aqueous solution when the degree of acetylation (DA) is larger than 40-50%; while, chitosan is a polysaccharide obtained by de-acetylation of chitin (Kumar 2000). Chitosan is composed of D-glucosamine residues (D-residue) along with N-acetylated residues, and becomes soluble in acidic conditions (Brugnerotto et al. 2001).

Solubility: The degree of acetylation is an important characteristic of chitosan because it determines the content of free amino groups in the polysaccharide. This property affects the solubility of chitosan (Pillai et al. 2009). Due to the amine groups, chitosan will be

soluble in dilute acidic solution because the amine groups become protonated and there is an electrostatic repulsion between the molecules (Brugnerotto et al. 2001). Chitosan may become insoluble at alkaline and neutral pH because the amine groups lose their charges, so there is no electrostatic repulsion keeping the molecules apart. Chitosan with a low degree of de-acetylation (40%) has been found to be soluble up to pH 9.0; whereas chitosan with higher degree of de-acetylation (~85%) is soluble only up to pH 6.5 (Ravindra et al. 1998). In addition, the ionic strength of the solution is also an important factor. Higher the ionic strength, the lower the solubility of chitosan. The addition of an electrolyte will reduce the electrostatic repulsion between the positively charged chitosan chains, and thus results in a salting-out effect leading to the precipitation of chitosan from the solution (Chang et al. 2015a).

Viscosity: The viscosity of chitosan solution depends on chitosan concentration, molecular weight, degree of acetylation, temperature, ionic strength and pH. Chitosan has high viscosity in an acidic solution due to its high molecular weight and linear unbranched structure. In general, as the temperature increases, the viscosity of the solution decreases. In addition, the viscosity of chitosan solution tends to increase with pH (Costa et al. 2015). The intrinsic viscosity of chitosan has been shown to be a function of its degree of ionization as well as ionic strength. At low degrees of acetylation, electrostatic repulsions between protonated amino groups are predominant, causing the expansion of molecules (Chang et al. 2015a; Costa et al. 2015; Lewandowska 2015). Schatz et al. has mentioned that the acetylated units can be involved in intramolecular hydrogen bonding and therefore the rotation around the (β-1→4) glycosidic linkages is limited. Thus, the stiffness of the chains should increase with degree of acetylation. The stiffness of the chain involves electrostatic repulsion and hydrogen bonding, therefore the ionic strength of the solution should also be a factor (Schatz et al. 2003).

Chitosan behaves differently in different solutions; for instance, chitosan behaves as a more compact sphere in aqueous acetic acid solution but as a random coil in urea. If the pH is adjusted using different anions, the solution viscosity will be different because of the screening of the protonated amino groups or steric hindrance exerted by the anion and the intramolecular electrostatic repulsion. For example carboxylate ions increase the chain stiffness of chitosan, whereas the chloride ion is too small to affect the chain stiffness. The viscosities of chitosan at pH between 2.0 and 6.4 are in the order of butyrate > propionate > acetate, in consideration of the anion

concentration (El-Kafrawy 1982). When the pH of the solution is increased, the intermolecular and intramolecular electrostatic repulsions between charges are decreased because of its amino groups. Therefore the chitosan chain can come closer and cause lowering of the hydrodynamic volume of molecules and viscosity of the solution. This may increase the intra-chain and inter-chain hydrogen bonding (Chang et al. 2015a; Costa et al. 2015; Lewandowska 2015).

Pectin

Pectin is a heterogeneous complex polysaccharide, extracted from plant cell walls, and its composition varies with source and the conditions applied during isolation. Pectin consists essentially of a linear chain of α-(l→4)-D-galacturonic acid residue interrupted by the insertion of (l→2)-L-rhamnopyranosyl units (Allwyn 2012). In addition, pectin can also contain some neutral sugars along its chain, such as galactose, glucose, rhamnose, arabinose, and xylose. Major applications of pectin in foods are thickeners, gelling agents, bakery fillings, glazing and stabilizers. Recently, pectin has been used for fat-substitution, and health benefits have been claimed (Allwyn 2012; Leroux et al. 2003; Thakur et al. 1997).

Molecular Structure: Pectin is mainly composed of polymerized, partly methanol esterified (1-4) - linked α-D-galacturonic acid. In some pectin, the methyl ester groups are partially converted to amide groups by using ammonia, called amidated pectin (Thakur et al. 1997). Commercial pectins are divided into “high ester” or “high methoxyl” pectin and “low ester” or “low methoxyl” pectin in accordance to the degree of methyl esterification (DE). The degree of methyl esterification is defined as the percentage of galacturonic acid units that is methyl-esterified (Allwyn 2012). Pectin with DE more than 50% is called “high-methoxyl pectin (HM-pectin)”. HM-pectin can form gel when the pH of the solution is low and a minimum soluble solids of 55% is reached. Pectin with DE lower than 50% is called “low-methoxyl pectin (LM-pectin)”. LM-pectin can also form gels; however, the gelation conditions are different from those required to form HM-pectin gels. LM-pectin gelation depends on the addition of calcium, which can be provided naturally by the fruit material or added as a separate ingredient (Allwyn 2012; Funami et al. 2011).

Physicochemical properties of solution: The physicochemical properties of pectin molecules in solution depend mainly on their degree of esterification, the arrangement of the methyl esters along the main chain, and the degree of polymerization; however, pH, ionic

strength, pectin concentration, presence of ions, water activity, and temperature also play important roles (Allwyn 2012; Funami et al. 2011).

Solubility: Pectin is usually soluble in water and insoluble in most organic solvents. As a general rule, the solubility is decreased with increasing ionic strength. There is no saturation limit for pectin but it is difficult to obtain true solutions with pectin concentrations higher than 3-4% (Chen et al. 2015).

Viscosity: The viscosity of pectin solutions depends on their concentration, presence of ions such as calcium, the degree of esterification (DE) and the average molecular weight (Schmidt et al. 2015). Dilute pectin solutions (below 0.5%) are Newtonian. Pectins have relatively low viscosities compared with other polysaccharides (Kar et al. 1999). The viscosity of pectin depends on the ionic strength of the solution. At high ionic strength, the viscosity of the solution decreases due to charge shielding; whereas at low ionic strength, the viscosity increase because of charge repulsion (Pals et al. 1952). Also in most cases, the viscosity increases with increasing pH (Evageliou et al. 2000). At concentrations higher than 1%, pectin solutions exhibit pseudo plasticity, such as viscosity decreases with increasing shear stress. In this type of solution, the viscosity of the solution increases with decreasing pH within the range of 2.5-5.5 in the absence of calcium (Marcotte et al. 2001). However, in the presence of calcium, the solution viscosity increases with increasing pH within the same range as mentioned before (Iglesias et al. 2004; Sato et al. 2008).

Polyelectrolyte behavior: Since pectin contains free carboxyl group on its main chain, it behaves as a polyelectrolyte. At neutral pH, dissolved pectin is negatively charged and approaches zero charge at low pH (Evageliou et al. 2000; Ngouemazong et al. 2015). Therefore, dissociation of the individual carboxyl groups is not independent. Because of its polyprotic acid nature, it is not possible to determine the p*K*a of pectin. Instead, the pKa of pectin depends on carbohydrate concentration and degree of dissociation (DD) (Thakur et al. 1997). The negative charge density depends on degree of esterification; thus, the higher the content of un-esterified galacturonic acid units, the higher the pKa. In addition, p*K*a is also dependent on polymer concentration and ionic strength, when the polymer concentration and ionic strength increase, p*K*a is lowered. In general, the pKa of pectin is in range of 2.9-3.3 (Ngouemazong et al. 2015; Nakauma et al. 2008).

The polyelectrolytic nature of pectin influences its rheological properties. At neutral pH, pectin in solution exhibits a random-coil conformation because of the

electrostatic repulsion between the charged groups. Conversely at low pH, pectin in solution changes its rheological property drastically and behaves differently from random coil behavior. Furthermore, when the ionic strength of the solution increases, the electrostatic repulsion is suppressed (Allwyn 2012; Sato et al. 2008).

2.3.3 Surfactants

Surfactant molecules are also used to form electrically charged oil droplets in oil-in-water emulsions. These molecules consist of a lipophilic tail group extended into lipid phase and hydrophilic group that extends to aqueous phase (Kim et al. 2008). Most available food grade ionic surfactant molecules induce anionic charge to droplets such as CITREM, lysolecithin and DATEM. Nevertheless, lauric arginate (LAE) is a positively charged surfactant molecule used to produce stable positively charged oil droplets at pH value less than 7.0 (Chang et al. 2015b). However, in many cases ionic surfactants are mixed with non-ionic surfactant molecules to enhance emulsion stability (Ibrahim et al. 2015).

2.4 Lipid Oxidation

Oils and fats begin to oxidize from the moment they are isolated from their natural environment resulting in qualitative deterioration of foods. Lipid oxidation follows two step process: primary oxidation and secondary oxidation (Berton-Carabin et al. 2014). Primary oxidation is a series of formation of hydroperoxides or peroxides via free radical mechanism. These flavorless and odorless transitionary intermediates are unstable, and decompose to generate secondary oxidation products which are volatile and small molecular weight compounds associated with development of off flavor in oxidative rancidity. Secondary products include carbonyl compounds, aldehydes, ketones, hydrocarbons, alcohols, and others. The more polyunsaturated the oil is, greater the free radicals generation, and successive reaction with oxygen by the lipid molecules (Chaiyasit et al. 2007). Comparative degrees of autoxidation of purified esters of linolenic acid ($C_{18:3}$): linoleic acid ($C_{18:2}$): oleic acid ($C_{18:1}$) were reported to be 25:12:1 on the basis of conjugated diene and peroxide development in matrices containing individual esters (O'brien 2008). Presence of antioxidant molecules and fatty acid composition of the oils are key factors which determines the susceptibility of oils to oxidation (Abramovič et al. 2007).

Rancidity in edible fats is a significant concern in food processing, particularly owing to consumer demands for increased use of polyunsaturated oils and reduced usage of synthetic antioxidants. In food applications, lipid oxidation is undesirable, yet unavoidable. In fact, only a small portion of the oil needs to be oxidized before it results in objectionable smell and taste and becomes unacceptable from sensory perspective (McClements et al. 2000). Lipid oxidation often leads to deterioration in processed food quality, including changes in flavor, color, and nutritional value, with the end result being reduced shelf life. Improvements in product shelf life have generally been achieved either by reducing the polyunsaturated fatty acid content through hydrogenation, genetic engineering or the outright replacement of an oil with less unsaturated counterparts. Other approaches such as oxygen exclusion from product during manufacturing, addition of antioxidants, and reduction of metal to control the production of free radicals have also been used (Chaiyasit et al. 2007).

2.4.1 Lipid oxidation mechanism

Lipid oxidation is a multifaceted free radical array reaction between oxygen and unsaturated fatty acids, which can takes place in an autocatalytic way. This reaction array in food and biological systems is driven by high-energy free radical species. These unstable radicals steal electrons and abstract hydrogen from lipids causing lipid oxidation. In general, lipid oxidation mechanism is categorized into three main pathways: initiation of free radical formation, propagation, and termination of accumulated free radicals.

In the initiation step, the first free radicals are formed. Potential pathways for free radicals formation are the reaction of Fe^{2+} with H_2O_2, the so called Fenton reaction, which is mainly considered in biological systems (Reaction 1), and also the Haber-Weiss reaction (Reaction 2-3), (Mahoney et al. 1986) where reducing agents convert iron to its more reactive ferrous state. However, in food systems, the reaction of Fe^{2+} and Fe^{3+} with existing lipid hydroperoxides (LOOH), a Fenton-like reaction, has been considered most important (Reaction 4-5). Several high-energy radical species such as hydroxyl radicals (OH•), lipid alkoxyl radicals (LO•), and lipid peroxyl radicals (LOO•) are generated from hydroperoxide decomposition. The fatty acid radical (L•) is generated by the removing of its hydrogen by these radicals (X•) (Reaction 6)

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$ (1)

$Fe^{3+} + O_2\bullet^- \leftrightarrows Fe^{2+} + O_2$ (2)

$2O_2\bullet^- + 2H \leftrightarrows H_2O2 + O_2$ (3)

$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO\bullet + OH^-$ (4)

$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO\bullet + H^+$ (5)

$LH + X\bullet \rightarrow L^{\bullet} + X\text{-}H$ (6)

Where X• represents LO•, LOO•, OH• and etc.

In the propagation step, lipid hydroperoxides (LOOH) and lipid peroxyl radicals (LOO•) are accumulated. These chain reactions originate from L•, which quickly reacts with O_2 to form LOO (Reaction 7) and then slowly turns into lipid hydroperoxides (LOOH) by hydrogen abstraction from another unsaturated fatty acid (Reaction 8). Most chain breaking antioxidants (AH) are able to inhibit the propagation process by inactivation of the longer lived lipid radicals such as LOO• and LO• (Reaction 9).

$L\bullet + O_2 \rightarrow LOO\bullet$ (7)

$LOO\bullet + LH \rightarrow LOOH + L\bullet$ (8)

$LOO\bullet + AH \rightarrow LOOH + A\bullet$ (9)

In the termination step, the accumulated radicals from the propagation step can be terminated by self-interactions to form non-radical species such as oxidized polar or non-polar dimers or trimers of lipids (Reaction 10-12).

$L\bullet + L\bullet \rightarrow L\text{-}L$ (10)

$LO\bullet + L\bullet \rightarrow LO\text{-}L$ (11)

$LOO\bullet + L\bullet \rightarrow LOO\text{-}L$ (12)

2.4.2 Lipid oxidation in emulsions

As discussed before, a typical oil-in-water emulsion has three components: (1) the internal oil phase, (2) the continuous phase in which the oil droplets are distributed and (3) the interfacial region made up of amphiphilic molecules referred to as emulsifier. The

interfacial region is of great importance with regard to oxidation of lipid phase in an oil-in-water emulsion, because this membrane might protect oil droplets from reactive species by acting as a barrier to the diffusion of molecular species and to penetration of components that initiates lipid oxidation in the oil droplet (Berton-Carabin et al. 2014). For example, free radicals are often more proliferated in the aqueous phase; therefore, it is necessary for the radicals to interact with the interfacial region before they can cause oxidation of lipids in the interfacial and the interior region. Oxidation, on the other hand, can influence the properties of the oil-water interface in the emulsion, especially in the later stages of oxidation (Nuchi et al. 2002). Many oxidation products possess surface active properties at the later stage of oxidation than the initial stage. These substances may diffuse into the water phase or even partition at the interface (Frankel 2014). The positioning of the lipid molecule at the interface of oil-water (e.g., perpendicular or parallel to the interface) may also be significant in lipid oxidation reactions because this will affect their availability to pro-oxidants present in aqueous phase (Chaiyasit et al. 2007; Ponginebbi et al. 1999).

Lipid oxidation in emulsions is affected by oil and emulsifier types and concentrations. The oxidation of emulsified oils varies from that of bulk oils, because of the existence of the interfacial membrane, the interactions between emulsifiers and other water soluble pro-oxidants at the interfacial region (Chaiyasit et al. 2007). The interfacial layer at oil-water influences the lipid oxidation process. This property is attributed to repel or attract antioxidants and pro-oxidant molecules by forming a physical barrier (Berton-Carabin et al. 2014). The reaction rate of lipid oxidation in polyunsaturated rich oil-in-water emulsion increases as the droplet size reduce due to greater exposure of surface area per unit volume to the pro-oxidants at the interface. However, due to action of emulsifiers and other factors, this may not occur (McClements et al. 2000).

Emulsions are destabilized as an effect of lipid oxidation, and it is thought that interactions among lipid hydroperoxides positioned at the oil droplet surface, and transition metals in the water phase are of most common origin of oxidative instability (McClements et al. 2000; Mei et al. 1999; Donnelly et al. 1998). Difference in the oxidative stability of emulsions stabilized using proteins may be due to differences in amino acid composition of proteins. Certain amino acids bind more powerfully to oil droplets, and can chelate more transition metals reducing lipid oxidation levels. However,

proteins can impart charge on oil droplets at pH value below or above their isoelectric points (Nielsen et al. 2004).

2.5 Factors Influencing Lipid Oxidation in Emulsions

The properties of the oil-water interface are determining factors responsible for the unique oxidation behavior observed in food emulsions. Though the presence of the interface generally promotes oxidation of emulsified oil, the judicious selection of an emulsifier may significantly counter this effect (Mancuso et al. 2000). Numerous studies have shown that the interface (and the corresponding increase in surface-to-volume ratio) increases the interactions among lipid hydroperoxides and aqueous phase pro-oxidants.

2.5.1 Aqueous phase composition

By definition, food emulsions are compositionally complex. The components potentially present in the aqueous phase include polysaccharides, sugars, proteins, surfactants, salts, acids, bases and buffer, all of which may positively or negatively influence lipid oxidation according to their chemical nature and local environmental conditions. It is therefore significant to ascertain the role of common ingredients used in preparation of food emulsions on lipid oxidation.

Polysaccharides and sugars: Polysaccharides are usually added to emulsions to increase continuous phase viscosity, which can improve emulsion stability by slowing droplet-droplet interactions. However, it has also been reported that polysaccharides can hinder lipid oxidation in oil-in-water emulsions through hydrogen donation, transition metal binding and free radical scavenging (Chen et al. 2010). Common polysaccharides such as low methoxyl pectin (LMP), xanthan gum, high methoxyl pectin (HMP), sodium alginate, methyl cellulose and α- carrageenan have been reported to hinder the rate of lipid oxidation via chelating metal ions (Shimada et al. 1994; Shimada et al. 1992; Matsumura et al. 2003). Gum tragacanth has been shown to act as a radical chain breaker by donating hydrogen (Shimada et al. 1992). Glycoproteins such as gum arabica and soy bean soluble polysaccharides have also shown to retard lipid oxidation via free radical scavenging by the protein fraction (Matsumura et al. 2003; Shimada et al. 1992; Shimada et al. 1994). Other studies have reported that sugars such as hexose, pentose, and reducing disaccharides can considered as strong pro-oxidants. Specifically, reducing sugars

stimulate lipid oxidation process while non-reducing sugars such as sucrose, hinder lipid oxidation (Sims et al. 1979; Yamauchi et al. 1984).

Proteins: Besides providing stability against droplet flocculation or coalescence, proteins also have been shown to hinder lipid oxidation through different mechanism, including free scavenging, metal chelation, inactivation of reactive oxygen species and hydroperoxide reduction (Elias et al. 2008). The antioxidant ability of a protein inherently depends on its chemical properties and the solvent accessibility of its amino acid residues (Huang et al. 1999; Nielsen et al. 2004). Biologically derived amino acids comprising either aromatic side chains (e.g., tyrosine, tryptophan, and phenylalanine) or nucleophilic sulfur-containing side chains (e.g., methionine, and cysteine,) have been shown to be active in inhibiting lipid oxidation (Elias et al. 2008). For example, the antioxidant activity of whey protein is thought to be due to the presence of sulfhydryl and non-sulfhydryl functional groups. A number of studies have reported that proteins may act as pro-oxidants under certain conditions whereas others such as heme proteins and lipooxygenases are pro-oxidants by nature (Allen et al. 1982; Tong et al. 2000a; Tong et al. 2000b; Donnelly et al. 1998).

Surfactants: Surfactants have direct influence on surface property of lipid dispersions and hence can affect oxidation. Besides charge related properties, emulsifiers may affect lipid oxidation by additional mechanisms (Berton-Carabin et al. 2014). Emulsifiers containing sugars (tweens) or amino acid moieties (proteins) may act as free radical scavengers and therefore retard lipid oxidation (Ponginebbi et al. 1999; Coupland et al. 1996a). While proteins can act as antioxidants, some proteins have shown to be prooxidants. The factors contributing to the antioxidant activity of proteins in oil-in- water emulsions may include their capability to scavenge free radicals, chelate metal ions, act as reductants (cysteine), and physically prevent interactions of aqueous phase components with interior lipids by forming thick cohesive membranes (Berton-Carabin et al. 2014). In addition, some proteins can act as antioxidative enzymes which inactivate peroxides and superoxides, while other may act as prooxidative enzymes which generate free radicals (McClements et al. 2000). Small molecule surfactants readily form a physical barrier at the oil-water interface in emulsions. When the interface is saturated, excess surfactant molecules will partition into the continuous phase and form surfactant micelles that may increase oxidative stability. For example, hemoglobin-catalyzed oxidation of emulsified sunflower oil was inhibited due to the presence of excess anionic surfactant in the continuous

aqueous phase (Sims et al. 1979). The authors inferred that the presence of negatively charged micelles interacted with the transition metals, thereby decreasing pro-oxidant level at the droplet surface (Cho et al. 2002; Nuchi et al. 2002; Richards et al. 2002).

Salts: As previously mentioned, salts can have a significant impact on the kinetic stability of oil-in-water emulsions. In regards to oil oxidation, salt may also screen electrostatic interactions between transition metals with the oil-water interface, thereby potentially increasing or decreasing their attraction with the interface. For example, addition of NaCl to negatively- charged SDS (sodium dodecyl sulfate)-stabilized corn oil emulsions showed a slight reduction in lipid oxidation rate in the absence of added iron whereas in the presence of iron, the lipid oxidation rate slightly increased (Mei et al. 1998b; Mei et al. 1998a). Different studies have suggested that depending on the system, salt could act as pro-oxidant or antioxidant (Osinchak et al. 1992).

2.5.2 Oil phase composition

The chemical structure and composition of lipids are significant factors dictating the susceptibility of oil to oxidation. As previously mentioned, saturated lipids are significantly more stable than unsaturated lipids against oxidation due to the absence of reactive sites (McClements et al. 2000). Differences in the molecular structure of unsaturated lipids in aqueous colloidal dispersions can show surprising results. For example, Miyashita et al. reported that unsaturated fatty acids solubilized in non-ionic surfactant micelles (polysorbate 20) showed increased oxidative stability with an increasing degree of unsaturation (Miyashita et al. 1994). In another study, Miyashita et al. demonstrated that when double bonds were closer to methyl end, fatty acids were more susceptible to oxidation (Miyashita et al. 1993). Coupland et al. showed that lipid oxidation was faster when triacylglycerols were located at the droplet surface rather than within the interior (Coupland et al. 1996b). Therefore, the polarity and surface activity of lipid molecules present within an emulsion droplet will strongly impact whether they will be located within the emulsion droplet or at its surface.

2.5.3 Emulsion droplet characteristics

The oxidation of emulsified lipids will vary depending on the concentration of emulsifiers, droplet size, physical state of the dispersed phase, and surface charge.

Droplet size: The typical diameter of emulsion droplets is 0.2-10 μm. The presence of a larger surface area (resulting from a smaller average droplet size) may lead to greater oxidation as the emulsified oil is potentially exposed to more aqueous phase pro-oxidants (Gohtani et al. 1999). However, other studies have reported that emulsion droplet size has an nonsignificant influence on oxidation rate. Gohtani et al. demonstrated that the oxidation rate of emulsified docosahexaenoic acid did not increase with a decrease in droplet size (Gohtani et al. 1999). This was likely due to the lack of reactive species present at the droplet interface.

Interfacial thickness: One of the significant characteristic of an emulsion matrix is its huge surface area. This fact suggests that the interfacial characteristics of the emulsion droplet might influence many reactions, including lipid oxidation. The thickness of a biopolymer interfacial layer can have a significant impact on the vulnerability of emulsified oil to oxidation. Layer thickness was invariably affected by the molecular weight and dimension of the biopolymer used. It has been reported that a casein-based interfacial layer (~ 10 nm thick) conferred enhanced stability against emulsified oil oxidation compared to a whey protein-based interfacial layer (1-2 nm) (Silvestre et al. 2000).

Surface charge: Lipid oxidation depends on the droplet charge status, with the type of emulsifier and pH of the medium impacting interfacial charge (Djordjevic et al. 2007). The surface charge of the dispersed lipid may greatly affect lipid oxidation rates. Ascorbic acid is approximately thousand fold less effective as an antioxidant in a negatively charged micellar system than in a positively charged micellar system (Mei et al. 1998b; Mei et al. 1998a). The differences in the effectiveness of ascorbic acid were attributed to electrostatic repulsion with the negatively charged micelles, which excluded the antioxidative ascorbic acid molecules from the region where oxidation takes place. Conversely, electrostatic attraction between ascorbic acid and the positively charged micelles allowed ascorbic acid molecules to concentrate where oxidation occurred resulting in more effective antioxidant protection. However, effect of interfacial charge on oxidation might be influenced by other emulsion inherent factors such as emulsion composition and emulsifier type. A number of studies have demonstrated that emulsions stabilized with anionic emulsifiers oxidize more rapidly than those stabilized with positively-charged emulsifiers. In the case of protein-stabilized emulsions, lipid oxidation has been shown to be faster at pH above the protein's isoelectric point (Silvestre et al. 2000; Mancuso et al. 1999). However, droplet charge does not necessarily equate with

susceptibility to oxidation. For example, Silvestre et al. demonstrated that casein-stabilized emulsions showed improved oxidative stability compared to whey proteins, though the latter had a higher droplet surface charge (+56 vs. +30 mV) (Silvestre et al. 2000). Other factors (amino acid profile, free radical scavenging ability) may also play a role in inhibiting lipid oxidation.

Available oxygen: There is always adequate oxygen exist in oil phase to initiate lipid oxidation as oxygen solubility in oil is three times higher than in water (Ke et al. 1973). As a result, an effective option to retard lipid oxidation is to reduce available oxygen concentration. It has been observed that at low oxygen concentrations, the rate-limiting step for the emulsified lipid oxidation was dependent on oxygen diffusion via the aqueous phase. The effect of low oxygen pressure on the kinetics of lipid oxidation in linoleic acid oil-in-water dispersion stabilized by Tween 20 has been studied. The rate of linoleic acid oxidation decreased as the temperature of the sample was increased due to decrease in the solubility of oxygen at elevated temperatures (Marcuse et al. 1968).

Presence of antioxidants: One of the most effective ways to retard lipid oxidation is to introduce antioxidants. The ‘polar paradox’ describes that hydrophobic antioxidants are more effective in emulsions than in oils and hydrophobic antioxidants are more effective in oils than in emulsions (Laguerre et al. 2015). The difference observed in the efficiency of antioxidants has been explained by their affinities toward the air-oil interfaces in bulk oil and the oil-water interfaces in the emulsions. The ability of an antioxidant to retard lipid oxidation depends on its concentration, polarity, physical location and environment (temperature, pH and ionic strength) (Frankel 2014). Different studies have demonstrated that both non-polar and surface-active antioxidants (TBHQ, α- tocopherol, δ-tocopherol and propyl gallate) effectively inhibit lipid oxidation in oil-in water emulsions (Mei et al. 1999; Heins et al. 2007a; Heins et al. 2007b). Antioxidants inactivate different types of pro-oxidants (reactive oxygen species, metals and enzymes) and scavenge free radicals generated at different stages of oxidation (Heins et al. 2007a). Though synthetic antioxidants are highly-effective (BHA, BHT, TBHQ), there is growing consumer demand for more naturally-occurring ingredients in processed foods (Chaiyasit et al. 2007). This has motivated the food industry to search for more 'label-friendly' alternatives such as certain biopolymers (proteins or polysaccharides), carotenoids, tocopherols and plant extracts as natural antioxidants.

Chapter 3

Materials and Methods

3.1 Materials

Pectin (Degree of esterification was reported to be 65.0-70.0% by the manufacturer), chitosan (Degree of deacetylation was reported to be ≥ 75.0% by the manufacturer), acetic acid, sodium chloride, sodium acetate, isooctane, methanol, butanol, ammonium thiocyanate, serous sulphate hepta-hydrate, barium chloride, hydrogen chloride, butylated hydroxyl toluene, thiobarbituric acid, trichlorocacetic acid of analytical grade were purchased from HiMedia, India. 1,1,3,3 tetraethoxypropane was purchased from HiMedia India. Cumene hydroxide was purchased from Sigma, India. Flaxseed oil was gifted from AAK Kamani Oil Pvt. Ltd, Mumbai, India. Milli-Q water (Resistivity 18.2 MΩ.cm) was used in the preparation of all buffer solutions.

3.2 Methods

3.2.1 Emulsion preparation

Sodium acetate buffer (50 mM) pH 4.0 was prepared to study the effect of sonication time (1.0, 2.0, 3.0, 4.0, and 5.0 Min), pectin concentration (1.0, 2.0, 4.0, 6.0, and 8.0 mg/mL) and sodium chloride (20, 40, 70, and 120 mM). Buffer pH condition (4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 8.0) was varied and used as continuous medium for pH effect studies. Sodium azide (0.02%) was used as antimicrobial agent in the emulsion preparation (Caution: Sodium azide is toxic). Sonication was carried out using probe ultrasonicator (Qsonica, Q700; USA)

In studies related to sonication time effect (1.0, 2.0, 3.0, 4.0, and 5.0 Min), sonication was conducted at 50 % amplitude, 0.5 duty cycle and pectin at a concentration of 10.0 mg/mL was used to stabilize 10.0 mg/mL of flaxseed oil at pH condition 4.0. These conditions were selected as the basic study to determine minimum sonication time required to get less droplet size emulsion. Under pectin concentration, sodium chloride and pH studies sonication was done for five minute with 50 % amplitude and 0.5 duty

cycle. Externally emulsions were cooled using ice bath to reduce the thermal effect of ultrasonication on emulsion formation.

In case of bi-layer emulsion preparation, biopolymer solution containing pectin was prepared separately by dispersing 5.0 mg/mL pectin into 50 mM acetic acid buffer (pH 4.0) and stirring for at least five hours to ensure complete hydration. A stock emulsion was prepared by ultrasonication (amplitude-50%, time- 5.0 min, duty cyce-0.5) of prepared biopolymers solution with 10.0 mg/mL of oil (pH 4.0). Thus prepared primary emulsions were used to prepare bi-layer emulsion by diluting different proportion of oppositely charged chitosan biopolymer solutions. The pH of the resulting emulsion was then adjusted pH 4.0 using glacial acetic acid. The developed flocs were disrupted using sonication at 35 % amplitude for 20 seconds with 0.5 duty cycle.

3.2.2 Mean droplet diameter

Dynamic light scattering (DLS) technique is the most frequently used technique for determining the size of submicron particles. It can determine size of colloidal suspensions and solutions (micro emulsions, micelles) (McClements 2015).Primary emulsion and bi-layer emulsion droplet size was quantified using a Zetasizer Nano (Malvern Instruments Ltd, USA). For the analysis, emulsions were diluted with same buffer pH to attain 1:100 dilutions to reduce multi-scattering effect and viscosity on measurements. The droplet size values are average of three separate samples.

3.2.3 ζ- potential

Electrophoretic light scattering (ELS) was used to characterize the ζ- potential or surface charge of colloids in solution. ζ- potential is useful in evaluating the charge stability of colloidal dispersions. A droplet in solution can acquire charge either by adsorption of ions present in solution, by ionization of its surface groups or due to difference in dielectric constant between particle and dispersing medium (McClements 2015). ζ- potential values of both pectin emulsion and pectin-chitosan bi-layer emulsion were measured using Zetasizer Nano (Malvern Instrument Ltd, USA). Prior to emulsion analysis, they were diluted using the respective acetate buffers. The ζ- potential values are average of three separate samples.

3.2.4 Color measurement

Color of prepared pectin primary emulsions was measured using a colorimeter (HunterLab, Model ColorFlex EZ, USA) and coordinates of CIE color systems were recorded using a D65/10º setting (Daylight 65 illuminant/10º observer). Prior to analysis colorimeter was standardized using a white color standard tile with the following tristimulus values: X= 80.49, Y= 85.64, Z= 92.25. The recorded L^*,a,b^* values were used to calculate Hue angle, and Whiteness Index of the emulsions using the following *Equations 3.1* and *3.2*.

$$Hue\ Angle = tan^{-1}(\frac{b^*}{a^*}) \quad (3.1)$$

$$Whiteness\ Index = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \quad (3.2)$$

L*, a* and b* represents the whiteness, red-green axis and yellow-blue axis in color space, respectively.

3.2.5 Emulsion stability

Droplet growth ratio was measured to analyze the change in droplet size over time at 55 ºC (primary layer emulsion) (Karadag et al. 2013). For the measurement of droplet growth ratio in primary emulsion, mean droplet diameter of emulsion after 60 min of sonication (D_0) was considered as reference. The mean droplet diameter measured on the seventh day (D_7) for primary pectin-stabilized emulsion was considered to evaluate the emulsion stability by droplet growth ratio method, which is represented in *Equation 3.3*.

$$Droplet\ Growth\ Ratio = \frac{[D_7 - D_0]}{D_0} \quad (3.3)$$

Droplet growth ratio of bi-layer emulsion was analyzed over time at 40 ºC. For the measurement of droplet growth ratio, mean droplet diameter of emulsion after 60 min of sonication (D_0) was considered as reference. The mean droplet diameter measured on the fourteenth day (D_{14}) for bi-layer emulsion was considered to evaluate the emulsion stability by droplet growth ratio method, which is represented in Equation 3.4.

$$Droplet\ Growth\ Ratio = \frac{[D_{14} - D_0]}{D_0} \quad (3.4)$$

Diluted emulsions (1:100) were used for the calculation of size index (*Equation 3.5*) (Harnsilawat et al. 2006). The spectral absorption of diluted emulsions was measured at 800 nm and 400 nm against de-ionized water as blank using a UV-Visible spectrophotometer (Systronics, Model-AU2701, India). Generally, light scattering in emulsion depends on refractive indices of the phases, particle size, and wavelength. Oil droplet with various diameter scatters light at different wavelengths and light scattering increases with increasing droplet diameter. By measuring the spectral absorbance at two wavelengths, size index is calculated by taking ratio of absorbance at 800 nm (A_{800}) and 400 nm (A_{400}) and it can be calculated as emulsion stability.

$$Emulsion\ Size\ Index = \frac{A_{800}}{A_{400}} \quad (3.5)$$

3.2.6 Environmental stress factors

***For Primary-pectin-stabilized emulsion*:**

- *Thermal treatment:* 5 mL of prepared emulsions was subjected to thermal treatment at 100 ºC for 20 min followed by cooling in running ice-cold water. After cooling, emulsions were kept at 27 ºC for 24 hours prior to analysis. Mean droplet diameter and ζ- potential were measured to assess the stability against thermal treatment (Liu et al. 2016a).
- *Freeze*-thawing: In freeze-thaw stability of emulsion studies, 5 mL of emulsion was stored at -18 ºC for 20 hours followed by 2 hours thawing at temperature 30 ºC in water. Mean droplet diameter and ζ- potential were taken into consideration to assess the stability of pectin emulsion as affected by pH (Liu et al. 2016a).

***For bi-layer emulsion*:**

- *pH stability:* The influence of pH on emulsion properties was determined by preparing a series of samples with aqueous phases of pH condition 3.5 and 5.0.

- *Temperature stability:* The influence of temperature on emulsion properties was examined. The thermal treatments involved placing emulsions in test tubes, incubating them in a water bath set at a fixed temperature (40 to 100 ºC) for 20 minutes, and then cooling them to room temperature in running ice-cold water. After cooling, emulsions were kept at 27 ºC for 24 hours prior to analysis.

- *NaCl stability:* The influence of sodium chloride on emulsion properties was examined by adding different amounts of NaCl (30 to 80 mM NaCl) to the emulsions before preparation.

3.2.7 Rheological properties

Rheological properties of pectin emulsions were carried out in Rheolab QC (Anton Paar India Pvt. Ltd). The shear rate was adjusted from 1 to 150 s^{-1} (primary emulsion) or 1 to 200 s^{-1} (bi-layer emulsion). Change in viscosity and shear stress was recorded at 25 °C. The developed flow curves were fitted to power law model (*Equation 3.6*) as a function of shear rate and shear stress to study the flow behavior.

$$\tau = K(\gamma)^n \qquad (3.6)$$

Where τ is shear stress (Pa), γ is shear rate (s^{-1}), K is consistency coefficient ($Pa.s^n$), and n is flow behavior index. MATLAB 2015(a) software was used for curve fitting with 95% confidence bounds to find unknown coefficients K and n from equation.

3.2.8 Oxidative stability evaluation

- *Measurement of peroxide value:* Emulsion (10 mL) were filled in capped tube and incubated in the dark at 55 °C for 7 days (primary emulsion) or at 40 °C 14 days (bi-layer emulsion).For measurement of peroxide value, method was adopted form Shanta and Decker (Shantha et al. 1994; Qiu et al. 2015). Briefly, every day an emulsion aliquot (0.3 mL) was taken for measurement and it was mixed with 1.5 mL of chloroform/methanol (3:1 v/v) solvent mixture and centrifuged at 3200 × g for 2 min. 200 µL of the upper solvent phase from the centrifuge tube was added to 2.8 mL of methanol/1- butanol (2:1v/v), followed by the addition of 15 µL of ammonium thiocyanate (3.94 M) and 15 µL of ferrous iron solution (prepared by dissolving 1.6 g of barium chloride in 50 mL of water and 2 g ferrous sulphate hepta-hydrate in 50 mL of water. These solutions are mixed slowly by adding 2 mL of hydrochloride (10 N). Barium sulphate precipitate was removed and clear solution of ferric chloride was collected). After 20 min of incubation in dark at room temperature, the absorbance was measured at 510 nm using a UV-visible spectrophotometer (Systronics, Model-AU2701, India). Calibration curve was prepared using cumene hydroperoxide to calculate the concentrations of peroxides.

- *Measurement of thiobarbituric acid reactive substances (TBARS):* TBARS was measured to quantify secondary oxidation products according to (Scheffler et al. 2009). Briefly, Emulsion aliquot (0.4 mL) was mixed with 0.8 mL of thiobarbituric acid-butylated hydroxytoluene solution [Thiobarbituric acid solution (TBA): 15% w/v trichloroacetic acid and 0.375% w/v thiobarbituric acid in 1.76 mL hydrochloric acid (12M) and 82.9 mL of deionized water. Butylated hydroxyl toluene (BHT) solution: 2% BHT in ethanol. TBA-BHT solution: 3 mL of BHT solution was mixed with 100 mL of TBA solution] and mixture was vortexed followed by heating for 20 min in boiling water. The samples were cooled to room temperature and then centrifuged at 8500 × g for 10 min. The absorbance of supernatant was measured at 532 nm. 1,1,3,3 tetraethoxypropane was used as the standard for the preparation of calibration curve.

3.2.9 Statistical analysis

All results were reported as mean ± SD of three measurements. Analysis of variance (ANOVA) analysis was performed to test for statistical significance between treatments using SPSS (v. 21, IBM, USA). Multiple comparison tests were performed using Tukey's test at p-value of 0.05.

Chapter 4

Results and Discussion

4.1 Pectin Stabilized Emulsion; Influence of Sonication Time, Pectin Concentration, Sodium chloride and pH

4.1.2 Results

4.1.2.1 Emulsion characteristics

Mean droplet diameter and ζ- potential of pectin-stabilized emulsion were selected under emulsion characteristics as shown in Fig. 4.1 and 4.2. Hue angle and whiteness index were considered under optical properties of emulsion (Table 4.1). Under different sonication time conditions (1.0, 2.0, 3.0, 4.0, and 5.0 min) the droplet mean diameter decreased from 634.4 ± 25.32 to 517.6 ± 18.25 nm, (Fig. 4.1a) in contrast to this, droplet size increased with increment in NaCl concentration (Fig. 4.1c) ($p<0.05$). At sonication time between 2 to 5 min mean droplet diameter was changed not significantly ($p>0.05$). Influence of pectin concentration on droplet mean diameter was analysed, which corresponded to 754.4 ± 27.68 and 555.8 ± 24.82 nm at lowest (1.0 mg/mL) and highest (8.0 mg/mL) pectin concentration, respectively (Fig. 4.1c)($p<0.05$). At lower sodium chloride concentration (20 and 40 mM) droplet mean diameter was changed not significantly ($p>0.05$) and with increment in concentration (70 to 120 mM) led to increase in droplet size from 641.5 ± 14 to 817.0 ± 12.80 nm (Fig. 4.1c)($p<0.05$). Droplet size changed significantly with pH condition of the emulsion matrix; higher z-average (557.7 ± 16.61) was recorded at pH 4.5.

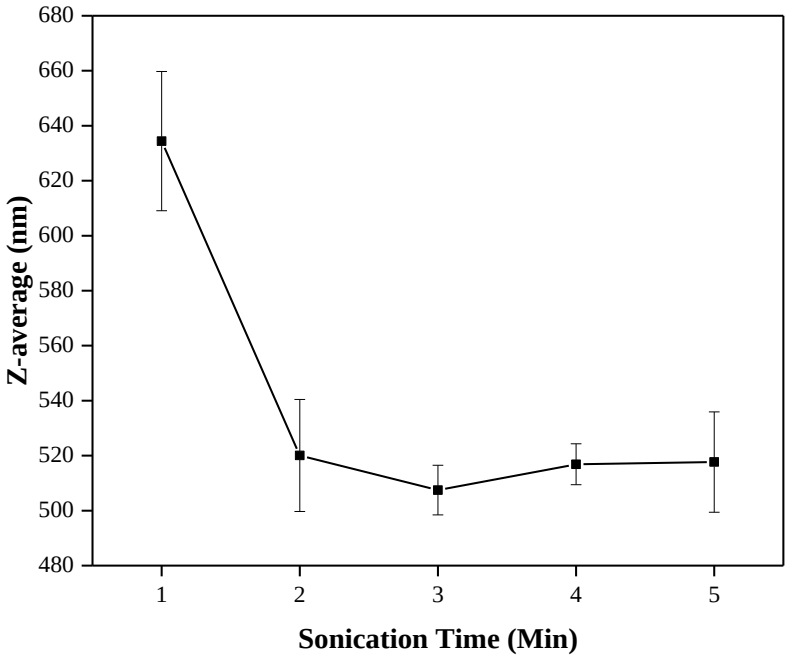
Fig. 4.2 represents ζ- potential (charge density) values of pectin-stabilized emulsions at different processing condition and continuous phase components. With respect to sonication time, no differences were found between charge density and sonication time at lower conditions (1 and 2 min) (Fig. 4.2a). However, increase in sonication time to 4 min decreased the absolute ζ- potential from -23.5 ± 0.67 to -22.1 ± 0.35 mV significantly. Significant charge density changes were recorded between different pectin concentrations (Fig. 4.2b). As a general rule, although the absolute value of ζ-potential differs between pectin concentrations, the sign of this difference is strongly

dependent on pectin adsorption at the interface. Under different sodium chloride concentration and pH conditions, charge density was significantly affected. In addition to a positive correlation of sodium chloride on charge density was observed. A large difference between measured values were recorded with respect to pH values (ζ- potential, NaCl 0 mM (pH 4.5 and sonication time 5 min), -23.1 ± 0.21 mV (Fig. 4.2a), and ζ-potential, pH 4.5, -11.0 ± 0.87 mV) (Fig. 4.2d), reflecting the combined effects of a greater capacity of sodium chloride to adsorb pectin at the interface and impart charge by having significantly same droplet mean diameter (Fig. 4.1c & d). Optical properties (hue angle and whiteness index) of pectin-stabilized emulsions measured after storage for seven days at two different temperatures (10 & 55 ºC) and fresh emulsion are tabulated in Table 4.1. Mean values for hue angle of fresh emulsion under different sonication time decreased significantly from 173.98 to 135.80º with increase in sonication time. At storage temperature 55 ºC hue angle in pectin emulsion reduced significantly irrespective of sonication time (Table 4.1a).

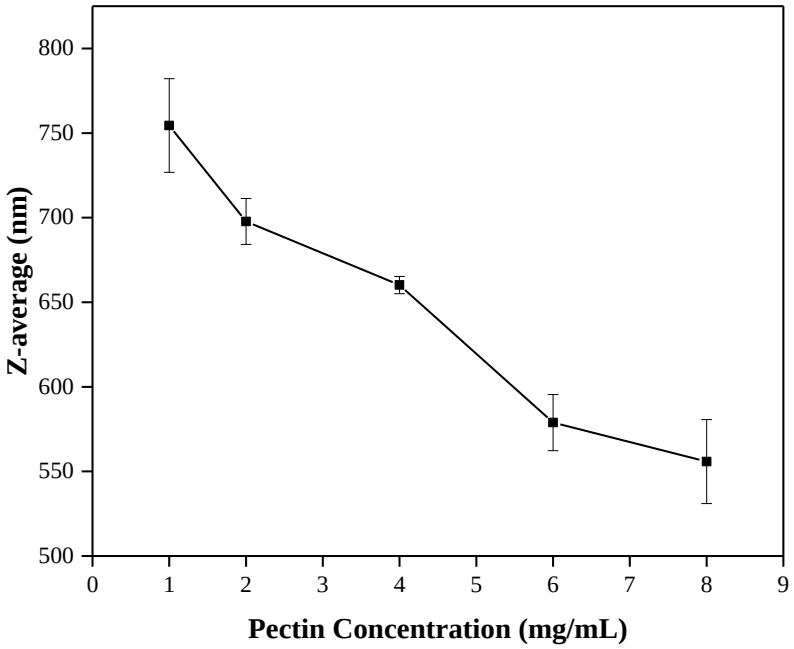
Storage of pectin emulsions at 10 ºC resulted in variation of hue angle significantly. At sonication time of 1 min, hue angle was 145.24 ± 0.89º and at 5 min it attained 133.26 ± 0.37º significantly (Table 4.1a). In contrast to this, hue angle increased apparently with increase in sodium chloride from 20 to 120 mM significantly. At 40 mM of sodium chloride concentration in pectin emulsion, 144.68º hue angles was recorded (Table 4.1c). In fact, no significant effect of sodium chloride on hue angle was recorded at lower concentration and during 10 & 55 ºC storage. However, above pH 5.5, pH had no effect on hue angle in fresh emulsions (Table 4.1d). At 55 ºC of storage, emulsion pH affected the hue angle significantly and it was in the range of 100.82 ± 0.59º to 107.90 ± 0.23º. Nevertheless, pectin concentration affected the hue angle differently compared to other parameters (Table 4.1b). At lower pectin concentration (1.0 to 4.0 mg/mL) hue angle was in third quadrant of color sphere irrespective of storage temperature. At pectin concentration of 8.0 mg/mL fresh emulsion had hue angle of 162.98 ± 0.22º. Storage at 10 & 55 ºC reduced the hue angle significantly to 147.09 ± 0.52º and 129.09 ± 0.78º, respectively (Table 4.1d). Whiteness index of the pectin emulsion was reduced to 59.46 ± 0.12 at sonication of 2 minutes. Highest whiteness index (72.46 ± 0.14) was recorded at 5 minute sonication (Table 4.1a). In case of variation of emulsion pH resulted in increase in whiteness index with increase in pH to 8.0. At higher storage temperature whiteness index

values reduced steeply in comparison with fresh emulsion and 10 ºC, in fact no significant effects were recorded (Table 4.1d).

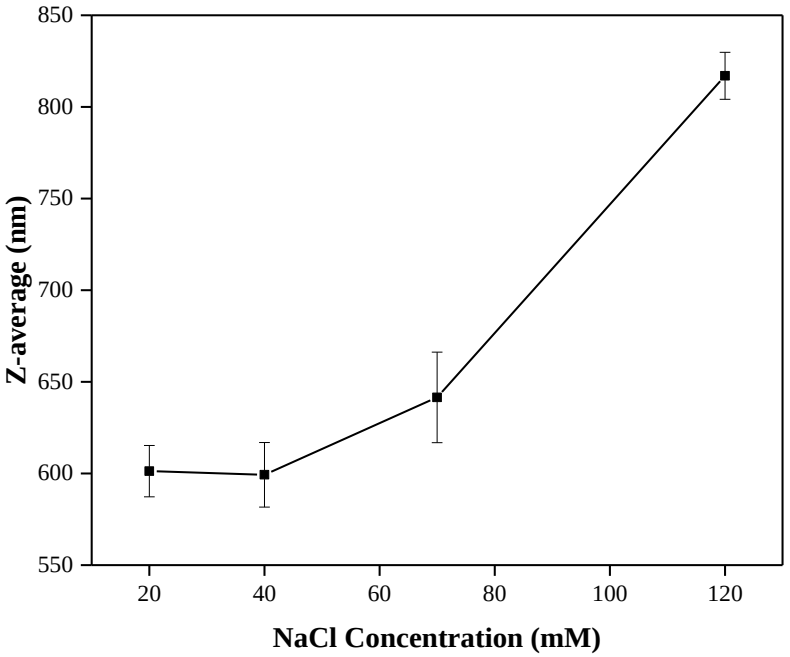
(a)



(b)



(c)



(d)

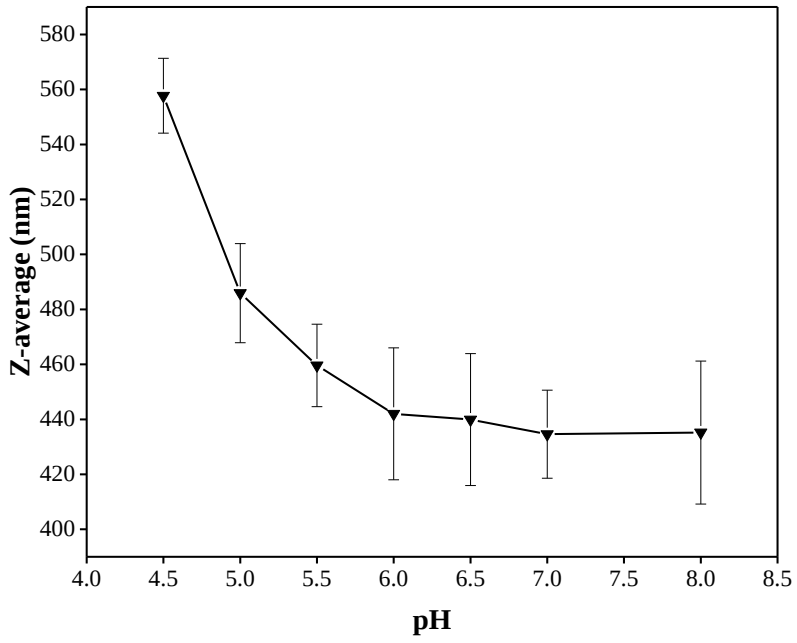
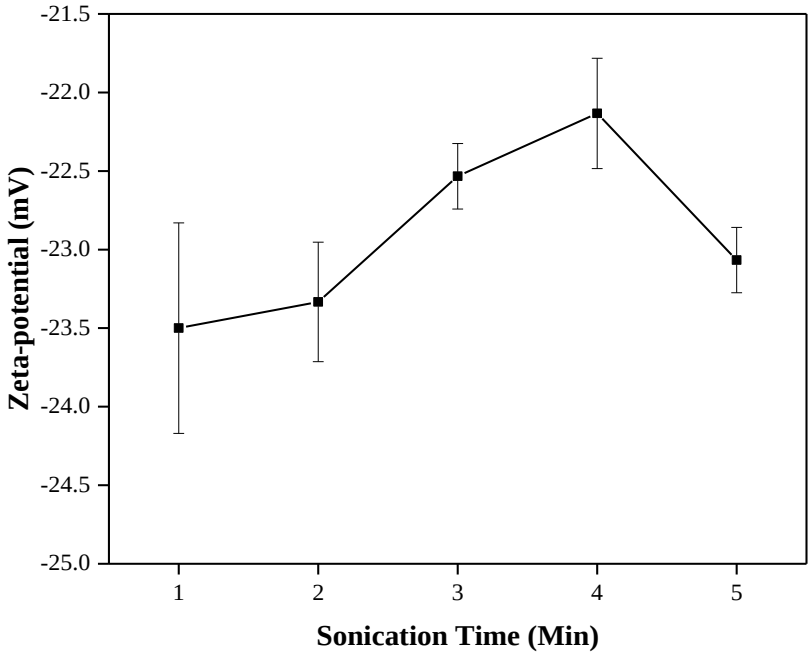
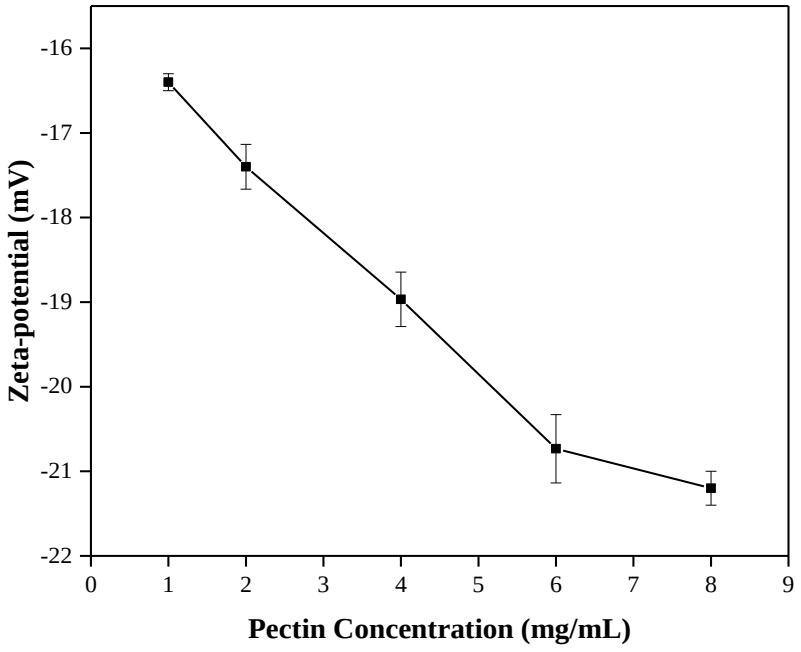


Figure 4.1: Influence of (a) sonication time (b) pectin concentration (c) NaCl concentration (d) pH on the droplets mean diameter of pectin-stabilized emulsion.

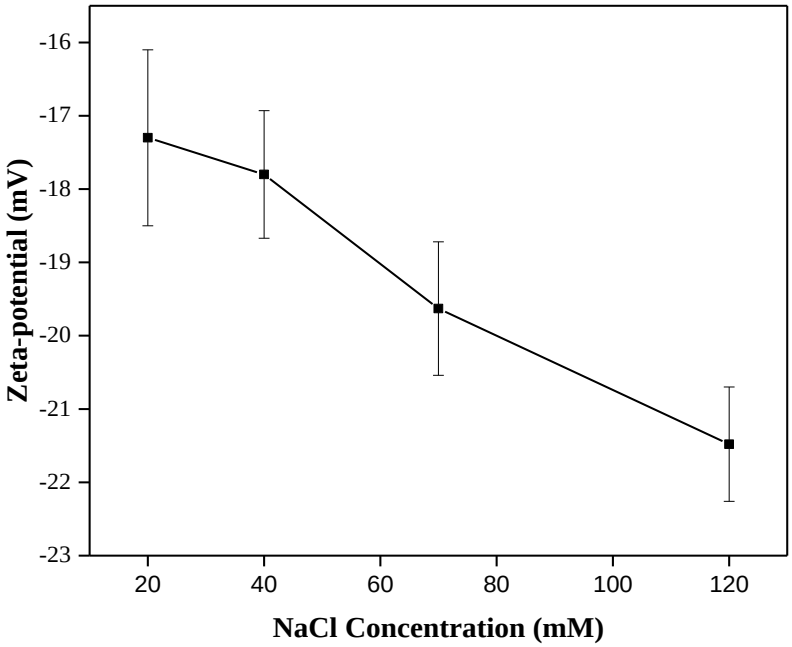
(a)


-21.5
-22.0
-22.5
-23.0
-23.5
-24.0
-24.5
-25.0
Zeta-potential (mV)
1
2
3
4
5
Sonication Time (Min)

(b)


-16
-17
-18
-19
-20
-21
-22
Zeta-potential (mV)
0
1
2
3
4
5
6
7
8
9
Pectin Concentration (mg/mL)

(c)



(d)

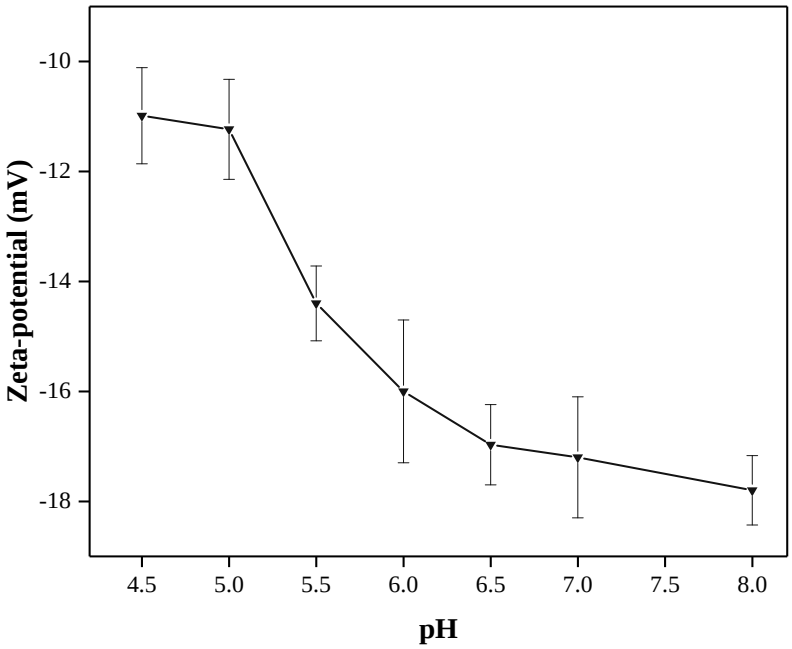


Figure 4.2: Influence of (a) sonication time (b) pectin concentration (c) NaCl concentration (d) pH on the ζ- potential of pectin-stabilized emulsion.

It was observed that, pectin concentration in the emulsion influenced whiteness index significantly. At lower pectin concentrations (1.0 to 4.0 mg/mL), emulsion whiteness index ranged from 59.80 ± 0.40 to 69.76 ± 0.02. Highest whiteness index (71.83 ± 0.02) was recorded at pectin concentration of 8.0 mg/mL, same condition was observed at 10 and 55 ºC storage (Table 4.1b). Increment in sodium chloride concentration significantly reduced the whiteness index of emulsion (Table 4.1c). At storage of 55 ºC and sodium chloride concentration of 120 mM, lowest whiteness index (68.75 ± 0.03) was obtained (Table 4.1c).

Table 4.1: Hue Angle and Whiteness Index of pectin-stabilized fresh emulsion during seven days of storage at 10 ºC and 55 ºC. (a) Influence of sonication time, (b) pectin concentration, (c) NaCl concentration, and (d) pH of emulsion.

(a)

Sonication time (Min)	Hue Angle (º)			Whiteness Index		
	Fresh Emulsion	7 days of storage at 10 °C	7 days of storage at 55 °C	Fresh Emulsion	7 days of storage at 10 °C	7 days of storage at 55 °C
1.0	159.35 ± 1.60^{c}	145.24 ± 0.89^{b}	117.15 ± 0.12^{a}	68.90 ± 0.25^{b}	70.04 ± 0.03^{c}	68.35 ± 0.02^{b}
2.0	173.98 ± 0.18^{d}	171.05 ± 0.39^{e}	129.44 ± 0.93^{d}	59.46 ± 0.12^{a}	59.57 ± 0.01^{a}	57.92 ± 0.01^{a}
3.0	154.63 ± 0.30^{b}	151.48 ± 1.00^{c}	121.76 ± 0.22^{b}	69.63 ± 0.09^{c}	69.64 ± 0.07^{b}	69.05 ± 0.14^{c}
4.0	161.67 ± 1.08^{c}	155.63 ± 1.28^{d}	124.70 ± 0.21^{c}	69.50 ± 0.11^{c}	69.60 ± 0.02^{b}	68.88 ± 0.07^{c}
5.0	135.80 ± 1.20^{a}	133.26 ± 0.37^{a}	117.14 ± 0.07^{a}	72.46 ± 0.14^{d}	72.36 ± 0.08^{d}	71.27 ± 0.06^{d}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(b)

Pectin concentration (mg/mL)	Hue Angle (º)			Whiteness Index		
	Fresh Emulsion	7 days of storage at 10 °C	7 days of storage at 55 °C	Fresh Emulsion	7 days of storage at 10 °C	7 days of storage at 55 °C
1.0	252.19 ± 0.96^{e}	249.81 ± 0.33^{e}	243.23 ± 0.65^{e}	59.80 ± 0.40^{a}	62.07 ± 0.10^{a}	61.45 ± 0.13^{a}
2.0	242.48 ± 0.97^{d}	243.94 ± 0.12^{d}	231.99 ± 1.74^{d}	65.33 ± 0.10^{b}	65.68 ± 0.02^{b}	65.18 ± 0.19^{b}
4.0	225.53 ± 0.45^{c}	212.84 ± 1.36^{c}	182.81 ± 1.11^{c}	69.76 ± 0.02^{c}	70.53 ± 0.01^{c}	69.34 ± 0.08^{c}
6.0	198.62 ± 0.39^{b}	182.65 ± 0.26^{b}	147.90 ± 0.87^{b}	70.51 ± 0.01^{d}	70.78 ± 0.05^{d}	69.71 ± 0.03^{d}
8.0	162.98 ± 0.22^{a}	147.09 ± 0.52^{a}	129.09 ± 0.78^{a}	71.83 ± 0.02^{e}	72.23 ± 0.17^{e}	71.59 ± 0.09^{e}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(c)

NaCl concentration (mM)	Hue Angle (º)			Whiteness Index		
	Fresh Emulsion	7 days of storage at 10 °C	7 days of storage at 55 °C	Fresh Emulsion	7 days of storage at 10 °C	7 days of storage at 55 °C
20	160.96 ± 2.16^{c}	141.58 ± 1.82^{a}	120.01 ± 1.55^{a}	70.92 ± 0.08^{c}	71.27 ± 0.03^{c}	69.19 ± 0.02^{b}
40	144.68 ± 1.34^{a}	139.25 ± 0.78^{a}	117.65 ± .48^{a}	71.47 ± 0.09^{d}	71.71 ± 0.01^{d}	70.57 ± 0.01^{c}
70	156.27 ± 0.49^{b}	148.12 ± 1.35^{b}	121.24 ± 0.66^{b}	70.47 ± 0.12^{b}	70.45 ± 0.01^{b}	69.33 ± 0.04^{d}
120	173.85 ± 1.67^{d}	153.25 ± 1.39^{c}	128.94 ± 0.90^{c}	69.09 ± 0.01^{a}	69.98 ± 0.02^{a}	68.75 ± 0.03^{a}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(d)

pH	Hue Angle (º)			Whiteness Index		
	Fresh Emulsion	7 days of storage at 10 °C	7 days of storage at 55 °C	Fresh Emulsion	7 days of storage at 10 °C	7 days of storage at 55 °C
4.5	128.77 ± 1.53^{d}	130.69 ± 1.13^{de}	107.90 ± 0.23^{d}	73.13 ± 1.20^{a}	72.83 ± 1.45^{a}	69.84 ± 0.79^{a}
5.0	124.15 ± 1.26^{c}	134.68 ± 1.52^{e}	105.02 ± 0.41^{c}	73.11 ± 0.90^{a}	72.75 ± 2.83^{a}	70.05 ± 1.19^{a}
5.5	119.72 ± 1.39^{b}	129.90 ± 2.69^{de}	102.45 ± 0.67^{b}	73.52 ± 1.19^{a}	72.72 ± 4.77^{a}	69.50 ± 1.03^{a}
6.0	115.47 ± 0.56^{a}	114.14 ± 0.71^{a}	101.44 ± 0.10^{ab}	74.66 ± 2.68^{a}	72.99 ± 0.80^{a}	71.21 ± 0.92^{a}
6.5	113.65 ± 0.85^{a}	125.71 ± 1.45^{cd}	101.98 ± 0.84^{ab}	74.85 ± 0.65^{a}	73.43 ± 0.39^{a}	70.29 ± 2.08^{a}
7.0	113.38 ± 1.59^{a}	120.23 ± 0.67^{b}	100.82 ± 0.59^{a}	74.92 ± 0.93^{a}	74.76 ± 5.06^{a}	68.43 ± 0.70^{a}
8.0	112.27 ± 0.34^{a}	123.18 ± 3.49^{bc}	100.75 ± 0.38^{a}	75.46 ± 1.55^{a}	74.25 ± 1.17^{a}	70.43 ± 1.13^{a}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

4.1.2.2 Emulsion stability

Droplet growth ratio and emulsion size index were measured for different sonication time, pectin concentration, sodium chloride concentration and emulsion pH condition to assess the stability shown in Table 4.2. Increasing sonication time of flaxseed oil-in-water emulsion resulted in a significant decrease in droplet growth ratio in storage of pectin emulsion at 10 ºC (1 min of sonication: 0.105 ± 0.007, 5 min of sonication: 0.007 ± 0.001). This was due to a significant reduction in droplet mean diameter and to a lesser extent to ζ- potential, which was independent of the sonication time (Table 4.2a). Mean of

droplet growth ratio decreased at low pectin concentration, although significantly lower levels were observed only at 10 ºC of storage (Table 4.2b). Sodium chloride induced a negative impact on droplet growth ratio. At higher sodium chloride concentrations droplet growth ratio was higher, in fact no significant differences were found at 10 ºC storage. At 55 ºC of storage, similar results were recorded with significant difference between the recorded values (Table 4.2c). Different pH conditions of emulsion prompted a positive effect on droplet growth ratio in both storage temperatures (Table 4.2d). The increased mean value of droplet growth was possibly due to reduced steric interactions and increased charge density above pH 6.0 (Fig. 4.2d).

The mean values of emulsion size index varied drastically under different sonication time treatment. At two minutes of sonication, highest emulsion size index (0.783 ± 0.010) was recorded in fresh emulsion (Table 4.2a). Increase in sonication time decreased the emulsion size index, indicating the instability in fresh emulsion. However, during storage at 10 & 55 ºC, emulsion treated for five minutes was found to be more stable to change in droplet size in response to temperature change. Similar results were recorded in pectin concentration variation. At five minutes of sonication treatment for emulsion containing 8.0 mg/mL pectin, size indices were 0.427 ± 0.002, 0.449 ± 0.001, 0.423 ± 0.004 for fresh emulsion, storage at 10 and 55 ºC, respectively (Table 4.2b). In contrast to droplet growth ratio of pectin emulsion at different sodium chloride concentration, size index revealed that, pectin is more stable to droplet growth during storage at higher salt concentration (Table 4.2c). This difference might be due to the fact that sodium chloride enhances steric interactions in the polysaccharides stabilized emulsions resulting in reduced droplet bridging (Ngouemazong et al. 2015). Table 4.2d represents the stability index measurements of pectin emulsion in response to pH condition. Despite of mean droplet diameter variation in response to emulsion pH condition in different storage regimes, size index measurement was not significant at 10 ºC. In contrast to this, fresh emulsion size index significantly varied with respect to fresh emulsion. However, as a result of protonation of pectin molecules at pH 8.0, lower size index was recorded during 55 ºC storage (Table 4.2d).

Table 4.2: Pectin stabilized emulsions stability by Droplet Growth Ratio and Emulsion Size Index as a function of time and temperature. (a) Influence of sonication time, (b) pectin concentration, (c) NaCl concentration, and (d) pH of emulsion.

(a)

Sonication time (Min)	Droplet Growth Ratio		Emulsion Size Index		
	10 °C	55 °C	Fresh Emulsion	7 days of storage at 10 °C	7 days of storage at 55 °C
1.0	0.105 ± 0.007^{b}	0.899 ± 0.066^{b}	0.441 ± 0.008^{a}	0.392 ± 0.005^{b}	0.383 ± 0.002^{c}
2.0	0.220 ± 0.021^{d}	1.003 ± 0.018^{b}	0.783 ± 0.010^{c}	0.436 ± 0.006^{d}	0.395 ± 0.006^{d}
3.0	0.156 ± 0.012^{c}	0.975 ± 0.076^{b}	0.514 ± 0.011^{b}	0.358 ± 0.001^{a}	0.352 ± 0.006^{b}
4.0	0.052 ± 0.005^{a}	0.916 ± 0.091^{b}	0.537 ± 0.013^{b}	0.405 ± 0.004^{c}	0.330 ± 0.003^{a}
5.0	0.007 ± 0.001^{a}	0.398 ± 0.037^{a}	0.441 ± 0.002^{a}	0.440 ± 0.001^{d}	0.396 ± 0.002^{d}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(b)

Pectin concentration (mg/mL)	Droplet Growth Ratio		Emulsion Size Index		
	10 °C	55 °C	Fresh Emulsion	7 days of storage at 10 °C	7 days of storage at 55 °C
1.0	0.210 ± 0.018^{d}	0.330 ± 0.027^{b}	0.397 ± 0.009^{a}	0.390 ± 0.004^{a}	0.340 ± 0.005^{a}
2.0	0.179 ± 0.013^{d}	0.325 ± 0.016^{b}	0.434 ± 0.006^{bc}	0.442 ± 0.005^{b}	0.393 ± 0.012^{b}
4.0	0.118 ± 0.014^{c}	0.458 ± 0.010^{c}	0.473 ± 0.005^{d}	0.479 ± 0.008^{c}	0.420 ± 0.002^{c}
6.0	0.065 ± 0.002^{b}	0.113 ± 0.006^{a}	0.442 ± 0.004^{c}	0.438 ± 0.001^{b}	0.410 ± 0.002^{c}
8.0	0.030 ± 0.003^{a}	0.085 ± 0.005^{a}	0.427 ± 0.002^{b}	0.449 ± 0.001^{b}	0.423 ± 0.004^{c}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(c)

NaCl concentration (mM)	Droplet Growth Ratio		Emulsion Size Index		
	10 °C	55 °C	Fresh Emulsion	7 days of storage at 10 °C	7 days of storage at 55 °C
20	0.092 ± 0.008^{ab}	0.168 ± 0.016^{a}	0.533 ± 0.024^{c}	0.437 ± 0.005^{b}	0.394 ± 0.003^{b}
40	0.083 ± 0.009^{a}	0.283 ± 0.021^{b}	0.451 ± 0.007^{b}	0.426 ± 0.003^{ab}	0.391 ± 0.006^{b}
70	0.094 ± 0.006^{ab}	0.305 ± 0.027^{b}	0.460 ± 0.004^{b}	0.442 ± 0.011^{b}	0.394 ± 0.001^{b}
120	0.112 ± 0.008^{b}	0.280 ± 0.024^{b}	0.406 ± 0.009^{a}	0.414 ± 0.002^{a}	0.367 ± 0.004^{a}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(d)

pH	Droplet Growth Ratio		Emulsion Size Index		
	10 °C	55 °C	Fresh Emulsion	7 days of storage at 10 °C	7 days of storage at 55 °C
4.5	0.012 ± 0.001 [a]	0.050 ± 0.003 [a]	0.652 ± 0.060 [ab]	0.662 ± 0.039 [a]	0.447 ± 0.030 [b]
5.0	0.026 ± 0.001 [a]	0.104 ± 0.008 [a]	0.634 ± 0.022 [ab]	0.624 ± 0.014 [a]	0.433 ± 0.019 [ab]
5.5	0.047 ± 0.001 [a]	0.152 ± 0.011 [a]	0.579 ± 0.041 [a]	0.573 ± 0.029 [a]	0.428 ± 0.011 [ab]
6.0	0.117 ± 0.001 [ab]	0.229 ± 0.008 [ab]	0.672 ± 0.014 [ab]	0.638 ± 0.025 [a]	0.456 ± 0.006 [ab]
6.5	0.166 ± 0.001 [b]	0.398 ± 0.013 [b]	0.703 ± 0.026 [b]	0.655 ± 0.040 [a]	0.468 ± 0.014 [b]
7.0	0.217 ± 0.001 [b]	0.346 ± 0.006 [b]	0.730 ± 0.035 [b]	0.602 ± 0.011 [a]	0.467 ± 0.002 [b]
8.0	0.207 ± 0.012 [b]	0.386 ± 0.031 [b]	0.740 ± 0.048 [b]	0.642 ± 0.100 [a]	0.420 ± 0.014 [a]

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

4.1.2.3 Emulsion stability to environmental stress factors

The mean diameter of droplets and ζ- potential responses of pectin-stabilized emulsions for thermal treatment and freeze thawing are summarized in Table 4.3 & 4.4. Overall results showed that a majority of all parameters (sonication time, pectin concentration, sodium chloride concentration and pH of emulsion) were affected by the thermal treatment.

In sonication time and pH parameters studies, pectin-stabilized emulsions appeared to be more stable to droplet growth (Table 4.3a & d). Additionally, the thermal treatment endorsed the clustering of pectin around the interface. At two and three minutes of sonication, pectin emulsion achieved 497.2 ± 8.03 and 456.7 ± 25.21 nm droplet sizes ($p>0.05$). In fact, compared to fresh emulsion (Fig. 4.2), absolute ζ- potential values were increased due to pectin alteration at the molecular level by thermal treatment (Ngouemazong et al. 2015; Allwyn 2012). The responses were similar amongst other parameters considered for stability assessment. Under pectin concentration variation, at low pectin concentration of 1.0 mg/mL, 1110.0 ± 16.10 nm was the highest mean droplet diameter recorded, after which there was a linear decline with increased pectin concentration (Table 4.3b). This phenomenon might be due to reduction of droplet flocculation induced by heating at higher pectin concentration at the interface.

The increased droplet size growth was always observed in different thermal treatment time and temperature. In case of different concentration of sodium chloride containing emulsions, thermal treatment affected the droplet size and ζ- potential. In fact,

there were no significant differences between the mean values with respect to charge density (Table 4.2c). There were significant droplet size and ζ- potential responses with the increased pH condition, which differed with fresh emulsion (Table 4.3 & Fig. 4.2). At lower pH conditions thermal treatment affected both mean droplet diameter and ζ-potential significantly. Above pH 5.5, charge density of the pectin-stabilized emulsions went linear with increase in pH (Table 4.3d). The droplet size of emulsion was 629.3 ± 35.32 nm at pH 4.5, which was the highest recorded value under thermal treatment in response to pH variation ($p<0.05$).

Table 4.3: Effect of thermal treatment on Z-average and ζ- potential of pectin emulsions. (a) Influence of sonication time, (b) pectin concentration, (c) NaCl concentration, and (d) pH of emulsion

(a)

Sonication time (Min)	Z-average (nm)	ζ- potential (mV)
1.0	696.4 ± 12.01^{c}	-16.6 ± 0.80^{ab}
2.0	497.2 ± 8.03^{a}	-15.9 ± 0.45^{b}
3.0	456.7 ± 25.21^{a}	-18.2 ± 1.21^{a}
4.0	540.0 ± 18.37^{b}	-15.8 ± 0.60^{b}
5.0	557.7 ± 7.05^{b}	-17.0 ± 0.70^{a}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(b)

Pectin concentration (mg/mL)	Z-average (nm)	ζ- potential (mV)
1.0	1110.0 ± 16.10^{d}	-13.8 ± 0.61^{b}
2.0	855.0 ± 09.02^{c}	-15.9 ± 0.83^{a}
4.0	803.6 ± 10.07^{b}	-16.3 ± 0.37^{a}
6.0	788.0 ± 13.82^{b}	-17.3 ± 0.98^{a}
8.0	671.0 ± 09.71^{a}	-17.6 ± 0.67^{a}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(c)

NaCl concentration (mM)	Z-average (nm)	ζ- potential (mV)
20	544.0 ± 15.04^{a}	-18.1 ± 0.82^{a}
40	694.6 ± 8.01^{b}	-16.8 ± 0.42^{a}
70	665.2 ± 18.38^{b}	-17.2 ± 0.35^{a}
120	691.2 ± 19.59^{b}	-17.0 ± 0.59^{a}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(d)

pH	Z-average (nm)	ζ- potential (mV)
4.5	629.3 ± 35.32^{b}	-16.3 ± 1.20^{c}
5.0	588.2 ± 42.11^{ab}	-18.2 ± 0.78^{bc}
5.5	581.0 ± 17.18^{ab}	-19.9 ± 1.40^{ab}
6.0	550.0 ± 48.04^{ab}	-21.2 ± 0.70^{a}
6.5	549.4 ± 12.68^{ab}	-22.2 ± 0.91^{a}
7.0	532.9 ± 24.48^{a}	-22.6 ± 1.10^{a}
8.0	519.0 ± 14.58^{a}	-21.8 ± 0.80^{a}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

By comparing droplet size and ζ- potential responses of different sonication time, pectin concentration, sodium chloride concentration and pH of emulsion under freeze-thawing treatment, we could determine whether pectin-stabilized emulsion was stable for thermal fluctuation during processing. At different sonication times, the droplet size (676.6 ± 13.07 nm) was less at five minutes of sonication ($p<0.05$). The mean values of charge density around the oil droplet also significantly varied in the range of -16.6 ± 0.51 to -18.3 ± 0.80 mV (Table 4.4a). This result was to be expected as response of relative droplet sizes in emulsion, pectin adsorption at the interface and amount of pectin in the continuous phase.

In pectin concentration variation experiments (Table 4.4b), at lower levels (2.0 and 4.0 mg/mL) the mean droplet diameter was higher than for the higher concentration levels. ζ- potential values were in the range of -14.8 ± 0.72 to -18.0 ± 0.57 mV (Table 4.4b). In

contrast to this, less droplet size emulsion was achieved at low sodium chloride concentration variation ($p<0.05$), with no significant change in ζ- potential of the emulsion (Table 4.4c). Droplet mean diameter and ζ- potential values were significantly varied in all experiments in which emulsion pH was varied (Table 4.4d). Pectin emulsion at pH 4.5, for example, had droplet size of 910.3 ± 41.41 and charge density of -19.00 ± 1.01 ($p<0.05$). Above pH 5.5, ζ- potential values decreased significantly with increased pH condition. In contrast to this, droplet sizes increased imparting more charge density around the droplet by reducing its surface area. However, this decreased ζ- potential value also might be due to reduced droplet concentration in the emulsion.

Table 4.4: Effect of freeze-thawing on Z-average and ζ- potential of pectin emulsions. (a) Influence of sonication time, (b) pectin concentration, (c) NaCl concentration, and (d) pH of emulsion.

(a)

Sonication time (Min)	Z-average (nm)	ζ- potential (mV)
1.0	933.5 ± 18.04^{d}	-17.2 ± 0.40^{ab}
2.0	879.1 ± 32.03^{c}	-17.3 ± 0.60^{ab}
3.0	741.3 ± 10.05^{b}	-16.6 ± 0.51^{b}
4.0	779.1 ± 15.02^{b}	-18.3 ± 0.80^{a}
5.0	676.6 ± 13.07^{a}	-17.3 ± 0.32^{ab}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(b)

Pectin concentration (mg/mL)	Z-average (nm)	ζ- potential (mV)
1.0	1445.2 ± 15.62^{d}	-14.8 ± 0.72^{c}
2.0	1133.3 ± 18.31^{c}	-15.9 ± 0.66^{bc}
4.0	1006.5 ± 14.87^{b}	-16.6 ± 0.48^{ab}
6.0	967.7 ± 12.19^{b}	-17.6 ± 0.84^{ab}
8.0	832.1 ± 16.22^{a}	-18.0 ± 0.57^{a}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(c)

NaCl concentration (mM)	Z-average (nm)	ζ- potential (mV)
20	695.6 ± 16.12^{a}	-16.4 ± 0.64^{a}
40	901.3 ± 19.20^{b}	-17.6 ± 0.81^{a}
70	903.1 ± 24.13^{b}	-16.4 ± 0.61^{a}
120	930.1 ± 36.71^{b}	-16.6 ± 0.72^{a}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(d)

pH	Z-average (nm)	ζ- potential (mV)
4.5	910.3 ± 41.41^{ab}	-19.0 ± 1.01^{c}
5.0	842.5 ± 31.04^{a}	-20.5 ± 0.60^{abc}
5.5	875.3 ± 14.81^{ab}	-22.0 ± 0.84^{a}
6.0	931.1 ± 24.04^{bc}	-21.6 ± 1.00^{ab}
6.5	867.2 ± 26.2^{ab}	-21.3 ± 0.74^{abc}
7.0	1006.0 ± 28.21^{c}	-19.2 ± 0.86^{bc}
8.0	924.1 ± 18.31^{b}	-20.7 ± 1.20^{abc}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

4.1.2.4 Rheological properties

Shear stress decreased with increase in sonication time; however, mean values of flow behaviour index was not significant ($p>0.05$). It was observed that increasing of sonication time reduced the consistency index due to decreased viscosity at higher shear rate values of pectin emulsion (Fig. 4.4a & Table 4.5a). At shear rate values between 0-50 s^{-1}, viscosity increased profoundly irrespective of sonication time. This property was attributed to high steric interactions between oil droplets at lower shear rate (Tadros 2015). Similar behaviour was observed at pectin concentration above 4.0 mg/mL and pH condition below 6.0. (Fig. 4.4b & c).

Above pH 6.0, pectin emulsion stabilization was more profound by the electrostatic interactions (Nakauma et al. 2008) and lowest consistency index (0.69 ± 0.03 $mPa.s^{n}$) was achieved at pH 8.0 ($p<0.05$) (Table 4.5c). Viscosity behaviour of an emulsion

stabilized at lower pectin concentrations was observed. Due to less steric interactions and reduced droplet concentrations in the emulsion matrix, flow behaviour of 1.94 ± 0.170 and consistency index of 0.03 ± 0.001 mPa.s^n was recorded. In fact, no significant mean differences were achieved at lower pectin concentrations (Table 4.5b).

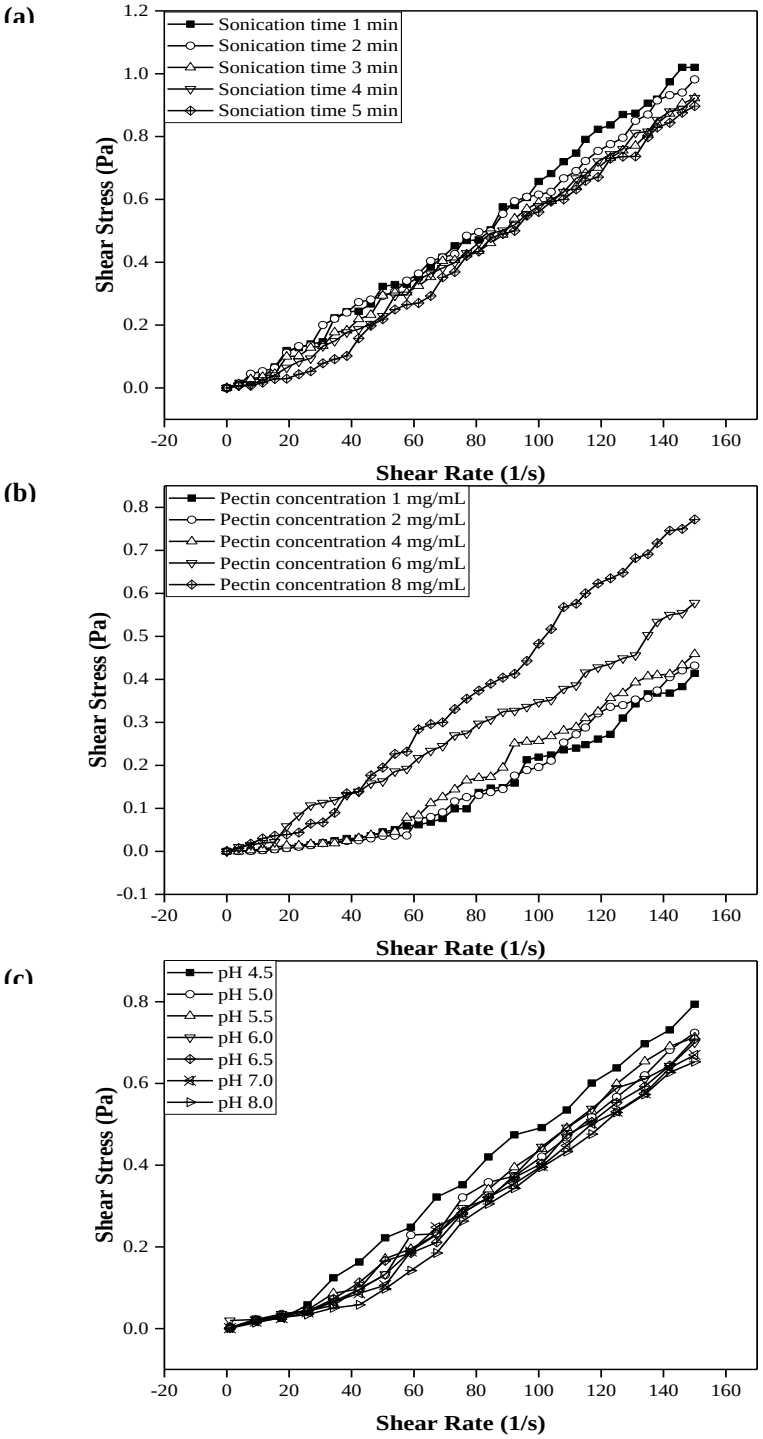


Figure 4.3: Influence of (a) sonication time (b) pectin concentration, and (c) pH on the shear stress of pectin-stabilized emulsion.

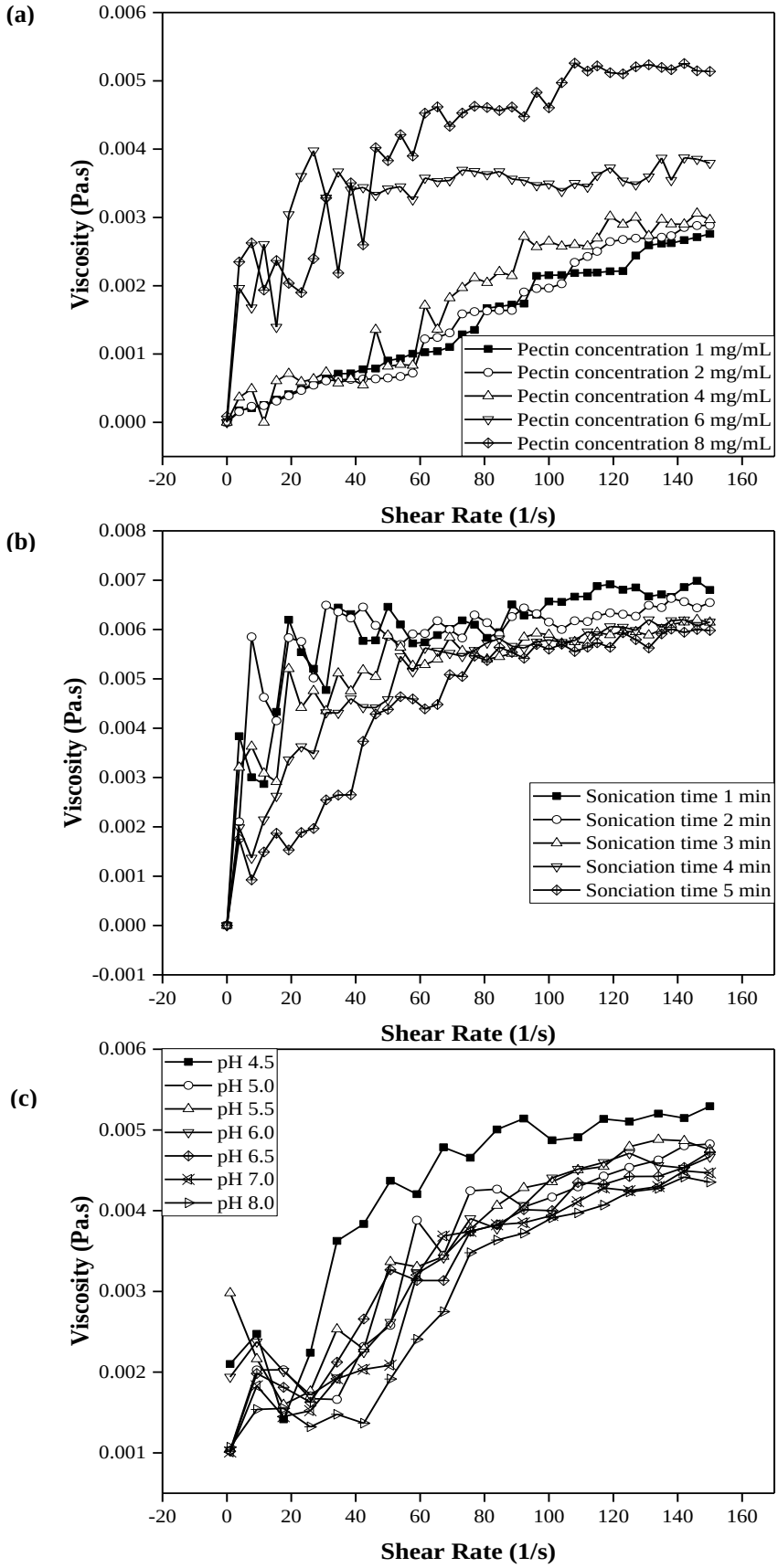


Figure 4.4: Influence of (a) sonication time (b) pectin concentration, and (c) pH on the viscosity of pectin-stabilized emulsion.

Table 4.5: Influence of (a) sonication time (b) pectin concentration, and (c) pH on the rheological properties of pectin-stabilized emulsion.

(a)

Sonication Time (Min)	Consistency Index, K (mPa.s^n)	Flow Behavior Index, n	Coefficient of determination (R^2)
1.0	4.25 ± 0.133^{d}	1.08 ± 0.018^{a}	0.9969
2.0	3.19 ± 0.040^{c}	1.15 ± 0.133^{a}	0.9975
3.0	2.92 ± 0.231^{c}	1.15 ± 0.110^{a}	0.9981
4.0	2.24 ± 0.172^{b}	1.21 ± 0.122^{a}	0.9978
5.0	1.27 ± 0.104^{a}	1.31 ± 0.081^{a}	0.9933

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(b)

Pectin Concentration(mg/mL)	Consistency Index, K (mPa.s^n)	Flow Behavior Index, n	Coefficient of Determination (R^2)
1.0	0.03 ± 0.001^{a}	1.94 ± 0.170^{c}	0.9920
2.0	0.02 ± 0.002^{a}	2.01 ± 0.210^{c}	0.9916
4.0	0.08 ± 0.002^{a}	1.73 ± 0.078^{bc}	0.9878
6.0	1.34 ± 0.104^{b}	1.28 ± 0.080^{a}	0.9948
8.0	1.37 ± 0.072^{b}	1.09 ± 0.072^{ab}	0.9941

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

(c)

pH	Consistency Index, K (mPa.s^n)	Flow Behavior Index, n	Coefficient of Determination (R^2)
4.5	2.11 ± 0.080^{e}	1.18 ± 0.050^{a}	0.9978
5.0	1.03 ± 0.040^{bc}	1.30 ± 0.016^{b}	0.9960
5.5	1.15 ± 0.023^{c}	1.28 ± 0.031^{ab}	0.9962
6.0	1.13 ± 0.032^{c}	1.28 ± 0.024^{ab}	0.9946
6.5	1.03 ± 0.041^{bc}	1.30 ± 0.037^{b}	0.9974
7.0	0.92 ± 0.017^{b}	1.31 ± 0.056^{b}	0.9961
8.0	0.69 ± 0.034^{a}	1.37 ± 0.041^{b}	0.9931

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$).

4.1.2.5 Oxidative stability

Peroxide values varied among the different sonication time, pectin concentration, sodium chloride concentration and pH condition over storage time of seven days at 55 ºC (Fig. 4.6). It was observed that during initial days (0-2 days) of storage, sodium chloride inhibited the development of peroxides. However, after initiation of lipid oxidation in emulsion matrix, development of peroxides was diminished up to 70 mM of sodium chloride. In addition, increasing of salt concentration to 120 mM resulted in increased rate of lipid oxidation (Fig. 4.6c). At one minute of sonication, pectin concentration (1.0 & 2.0 mg/mL) and pH condition above 6.5, lipid oxidation termination was witnessed (Fig. 4.6a, b & d). This might be due to generation of less number of droplets during less time of sonication. In addition, insufficient pectin at the interface and more negative charge around the droplet above pH 6.5 also responsible for increased oxidation (McClements et al. 2000). Five minutes of sonication enabled the lipid oxidation reduction, by adsorbing more pectin at the interface and development of more oil droplets. High pectin concentration at the interface covered the oil droplet sufficiently and prevented the contact with hydrophilic prooxidants present in continuous water phase. Adjusting the emulsion pH from 4.5 to 7.0 resulted in a 2.7-fold increase in peroxides. Higher viscosity of emulsions at lower pH condition (pH<5.5) prevented droplet aggregation. It reduced peroxide generation at the interface by restricting the droplet and prooxidant displacement.

Secondary lipid oxidation products were measured in terms of thiobarbituric acids reactive substances (TBARS). Fig. 4.7a shows that, for pectin stabilized emulsion, oxidation was greater and more rapid at one and two minutes of sonication than at five minutes of sonication. In pectin stabilized emulsion at less sonication time, TBARS formation increased throughout the entire incubation period, while TBARS formation was nearly constant in pH variation for the first two days after which formation rates increased (Fig. 4.7d). Initial pectin stabilized emulsion oxidation rates were faster at all sodium chloride concentration (Fig. 4.7c). TBARS concentration (Fig. 4.7b) increased throughout the oxidation period at lower pectin concentration. Increased rate of lipid oxidation in emulsions suggests that the pectin concentration was low and anionic charge present around the droplet enhanced the oxidation rate.

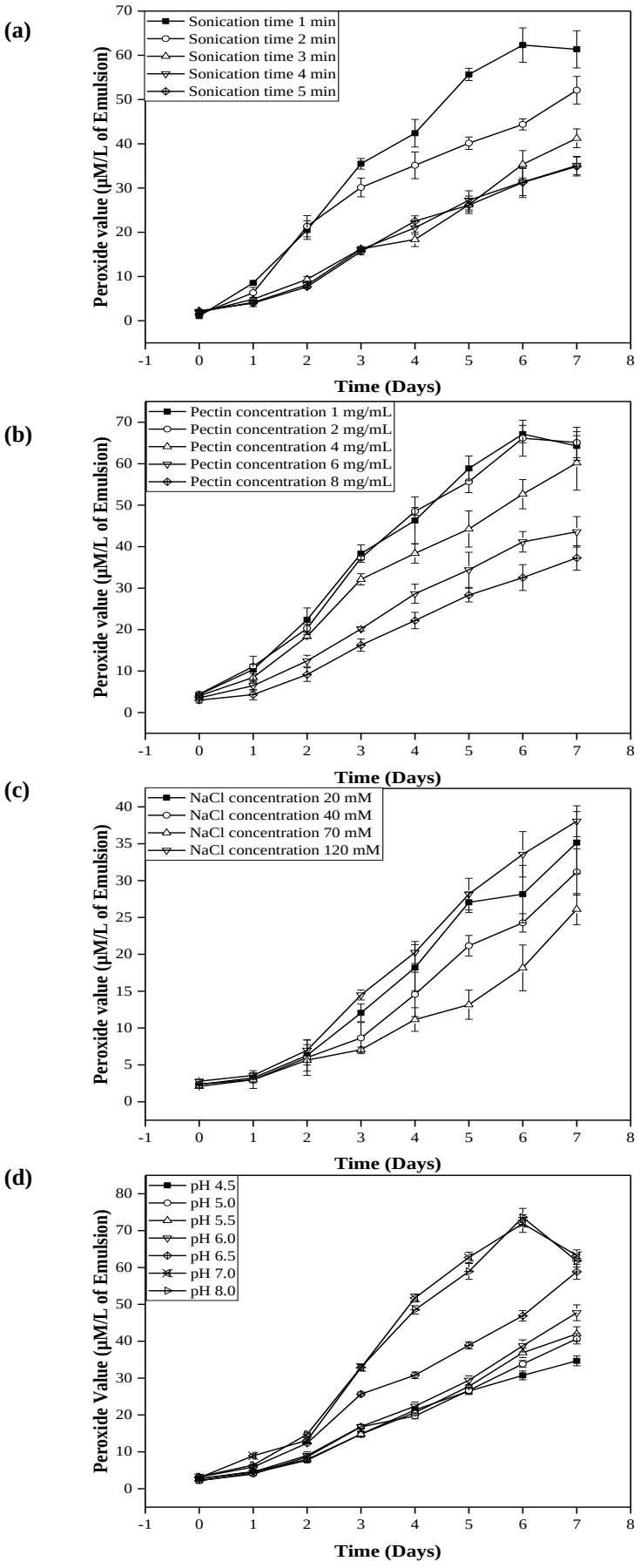


Figure 4.5: Influence of (a) sonication time, (b) pectin concentration, (c) NaCl concentration, and (d) pH of emulsion on peroxide value of pectin-stabilized flaxseed oil-in-water emulsion at 55 ºC

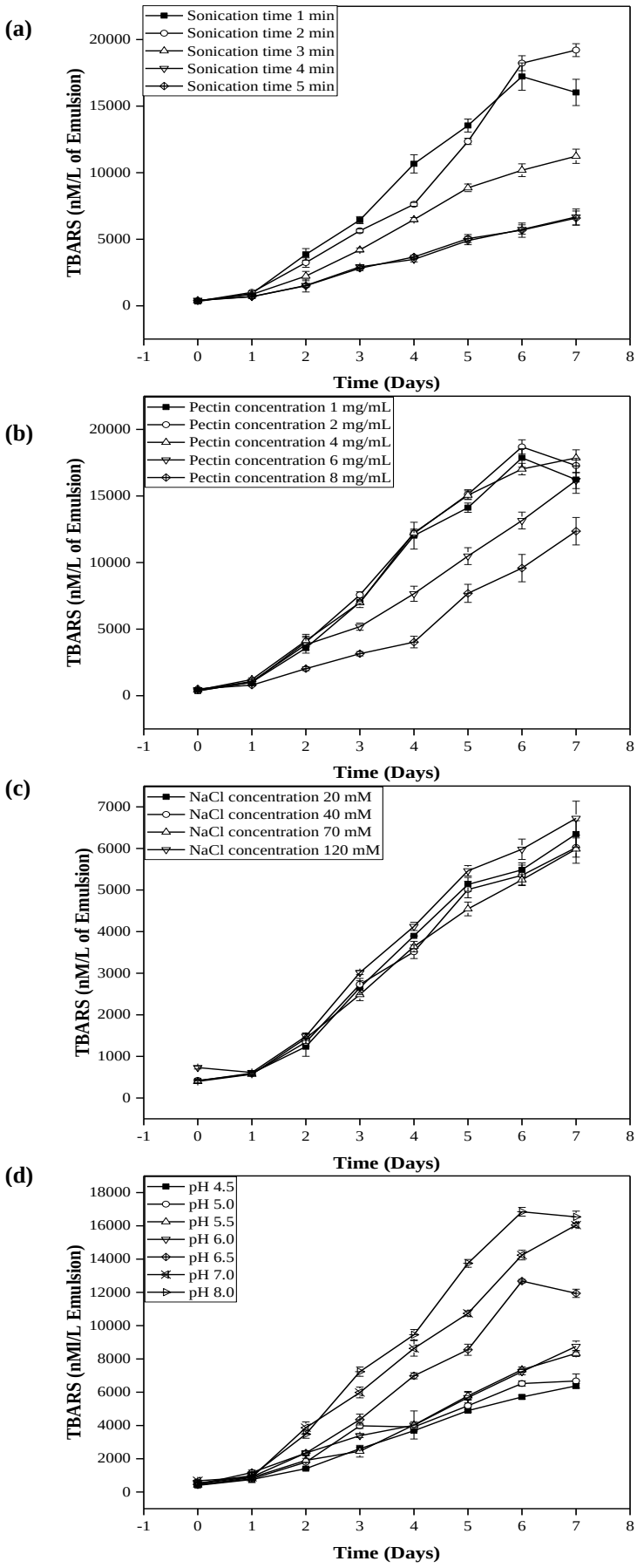


Figure 4.6: Influence of (a) sonication time, (b) pectin concentration, (c) NaCl concentration, and (d) pH of emulsion on TBARS of pectin-stabilized flaxseed oil-in-water emulsion at 55 ºC

4.1.3. Discussion

4.1.3.1 Emulsion characteristics

Emulsions were studied at different sonication time, pectin concentration, sodium chloride concentration and pH to investigate the potential relationship between droplet size and emulsion composition produced by ultrasonication. The present study clearly demonstrated that the variation in sonication time and emulsion composition affected the physical properties of pectin-stabilized emulsion. It is well known that emulsion stabilized by polysaccharide droplets were principally governed by steric mechanism and are therefore extremely prone to changes in solution pH (Chang et al. 2015a; Liu et al. 2009; Laplante et al. 2005; Evageliou et al. 2000). The physical stability of emulsions during manufacture, transportation, utilization and storage is a critical aspect for their practical application. Deviations in temperature can affect the stability of emulsion through different mechanisms. Heating leads to an increase in the droplet collision frequency which can promote aggregation under conditions where there is no strong repulsion between the droplets (Liu et al. 2016a). The change in droplet size is attributed to apparent viscosity of the emulsions, where viscosity of the solution plays a major role in changing emulsion droplet size in ultra-sonication assisted emulsification.

In any type of emulsion, processing parameters such as emulsification time and temperature plays a major role in determining homogeneity of oil droplet distribution in the continuous phase (Mahdi et al. 2006). In our current study, sonication time was fixed for 5 min and variation in emulsion composition influenced the homogenous distribution of flaxseed oil in pectin solution. Based on the interaction between viscosity and applied sonication power, droplet sizes were reduced and further dispersed in the continuous phase. Reduction of droplet size witnessed with increase in sonication time. This distinct effect of sonication was observed previously by other researchers and it was concluded that the increased dissipation of ultrasonic power in emulsion liquid decreases Laplace pressure with increase in temperature, which leads to instabilities to generate lesser diameter droplets (Kentish et al. 2008; Ramisetty et al. 2015). As an end result, concerning ultrasonic emulsion, a reliable optimum time level will have to be established to avoid the droplet size increase due to high rate of cavitation. It is also noticeable that at constant sonication power, increase of sonication time affects oil droplet size reduction referred to as “over-processing”. Similar trend was observed in earlier ultrasonication

emulsification and the effect was due to high shear rate and Bjerknes forces in emulsions (Kentish et al. 2008; Mahdi et al. 2006; Tornberg 1980).

Results demonstrated that in all sonication times, charge density varied. The absolute value of the electrical charge strongly depends on the type of polysaccharide used in the emulsion formation. As noted above, exception to this general pattern was that at five minutes the absolute ζ- potential value was highest. ζ- potential of the pectin emulsion increased with increase in pH significantly ($p<0.05$), however when pH was close to neutral (pH 6.5-8.0), charge density increased significantly compared to pH at 4.5-6.0 ($p<0.05$). This is possibly due to the alteration of pectin emulsifying activity because of modifications in the polysaccharide chain at the molecular level. However, during pectin adsorption at the oil-water interface, dominance of electrostatic repulsions (at higher pH) over attractive van der Waals forces stimulates emulsion stabilization through repulsive interactions between the droplets (Ngouemazong et al. 2015). The sharp decrease in size of emulsion droplet as pH increases above 4.5 is due to increase in negative charge on pectin (p*K*a ~ 2.9-3.3). In addition, in lower pH condition more than 90% of the free carboxyl groups in pectin are ionized, while at higher pH condition all free carboxyl groups are ionized. From the ionization status, a higher pH would be between for pectin-chitosan interaction but at pH ≥ 5.0 pectins are no longer stable and start to degrade. Pectin chain degradation occurs due to β-elimination, whereby the glycosidic bonds of the pectin backbone are cleaved, consequently reducing the molecular weight (Wüstenberg 2014). In general, the charge density on an oil droplet is related to stability of the emulsion. For a stable emulsion, the force of repulsion between droplets is essential. Sodium chloride influence on charge density can be explained by ability of chloride to form the complex in continuous phase.

The color of a beverage or any other food matrices depends on its components. The visual perception of any emulsion depends greatly on the dispersing medium and interface stabilizing material (McClements 2002). Hue angle is the polar representation of angular components. In general, hue color aspects are easier to conceptualize than color coordinates (a* and b*). At any horizontal cross section of the color sphere, all hues are represented in a 360º circle. A sample with a hue angle of 0º is purplish-red, 90º is yellow, 180º is bluish-green and 270º is blue (Chantrapornchai et al. 1998).

In emulsion, the major component includes oil phase, continuous phase and interfacial membrane that separate the two phases with the help of amphiphilic emulsifiers. The extent of light scattering by an emulsion generally increases as the droplet concentration is increased. However, the perceived emulsion light intensity may increase steeply with increase in droplet concentration and then reduce after certain droplet concentration is exceeded due to multiple-scattering effect (Chantrapornchai et al. 1998; McClements et al. 1998). The increased light intensity also depends on the ratio of droplet radius and wavelength of light. When droplet radius reaches above 100 nm, lightness decreases as the droplet radius is increased further (McClements 2002).This is due to difference in refractive index of oil droplet and continuous phase (Chantrapornchai et al. 1998). However, pectin as interface stabilizing polysaccharide also contributed to the development of color in flaxseed oil-in-water emulsion.

Hue angle of emulsions at low pectin concentration fell under the third quadrant of color sphere. This can be due to more bluish-green color of the emulsion. However, during storage, yellowness was developed in the emulsions and the hue angle was reduced significantly. In case of measured emulsions whiteness index, it increased up to a constant droplet concentration and it also depended on droplet size. In case of sonication time variation, pectin emulsion whiteness index increased with increase in sonication time. It should also be noted that highest absorbance of emulsion droplets will be near the incident light wavelength (Chantrapornchai et al. 1998). In general, under sonication-assisted emulsification, the energy produced from sonotrode will be transferred to oil droplets through continuous phase (McClements 2010). During this transfer, energy is also utilized to decreases the viscosity of the emulsion thereby helping to generate more number of oil droplets. However, increased sonication time may also disrupt the droplets causing coalescence, which results in lesser whiteness index due to multiple light scattering effects (Mahdi et al. 2006).

4.1.3.2 Emulsion stability

Droplet growth ratio and spectroscopy-based emulsion size index were considered as factors to determine pectin emulsion stability. An emulsion is considered stable when the difference in droplet size is not significant between a certain range of time. Factors including steric and electrostatic effects are considered as major factors for emulsion stability. The steric stabilization property of a polysaccharide is the result of

colonization of water molecules in emulsion between oil droplets (Funami et al. 2011). This colonization is boosted by osmotic pressure gradient and hydrated polysaccharide layer at the interface formed by extending into the continuous phase as a physical barrier (Ngouemazong et al. 2015).

The adsorption behavior of polysaccharide dispersion depends on pH of emulsions to which protonation of the anionic carboxylic group relates (Thakur et al. 1997). The interface stabilizing property of pectin was hardly affected by the change in pH value, and sodium chloride concentration; however change in temperature influenced the stability during storage. Droplet growth had positive correlation with both storage temperatures in all the sonication time and emulsion composition variation. However greater instability was observed at storage temperature of 55 °C. This can be explained by the fact that at higher temperature, increased movement of droplets results in droplet collision and leads to instability due to coalescence. Based on the viscosity of the emulsion, droplet movement can be understood. It can be observed that at all pH levels, the emulsion size index decreased with increasing storage temperature. This decreased size index values are evident for the droplet growth ratio increase at higher temperature. The spectral reflectance increased with droplet radius. Higher droplet growth ratio infers the low stability of emulsions. Phase separation was inhibited due to strong steric interactions between the droplets (Ngouemazong et al. 2015).

The scattering efficiency can be achieved when diameter of droplet is approximately equal to the wavelength of incident light. Above this point, with increasing droplet diameter, reflectance reduces as the scattering of dispersion phase decreases (Chantrapornchai et al. 1998; McClements et al. 1998). However at different emulsion composition, the emulsion destabilizing factors became stronger during storage and generates transient network that retards or even inhibits phase separation over the storage period. This is due to strong inter-droplet forces, which prevents droplet rearrangement and expels the serum phase from the structure during storage. Decrease in emulsion stability index over time and at high temperature was ascribed to droplet growth at high temperature. In addition to this, at high temperature, increased droplet collision in continuous phase also resulted in droplet flocculation.

4.1.3.3 Environmental stress factors

Studies related to influence of environmental stresses can be connected to long term stability and different thermal process parameters on emulsion properties. Emulsions may undergo heating, freezing and thawing treatments during processing, storage and consumption. The focus of the experiments was to examine the influence of thermal processing and freeze-thaw stability on emulsion properties. When an emulsion was cooled to temperatures where only dispersed oil phase became partially crystalline, the polysaccharide stabilized emulsions were generally stable (Degner et al. 2014). These properties are attributed to the ability of polysaccharides to form thick interfacial membrane around oil droplets that is difficult to penetrate by ice crystals from the continuous phase. Nevertheless, when temperature is reduced below sub-zero temperature, the interfacial membrane becomes susceptible to disruption triggered due to further destabilizing effect of the crystalline water in the continuous phase (Liu et al. 2016b). Hence the instability can be ascribed to crystallization of emulsion components. When emulsions were thawed, the droplets tend to aggregate, resulting in phase separation due to freeze-thaw induced coalescence. During heat treatment, oil droplets in emulsion systems move faster than ambient temperature causing instabilities such as coalescence, flocculation, aggregation, and in some cases gelling (caused due to swelling of hydrated interfacial layer and excess polysaccharide in continuous phase). Many studies found polysaccharides could contribute to the stability against environmental stress factors. In order to better understand this point, pectin emulsions underwent the process of heating and freeze thawing. After these treatments, the change in emulsion droplet size and ζ-potential of pectin emulsions were examined.

The aggregation of droplets causes a change in the density difference depending on the compaction of the flocculation. Therefore, it would affect the distribution of the electric field around the oil droplet. The relaxed the flocculation, the more the field lines will be able to penetrate, thereby reducing the tangential electric field at the outer surface of the floc (McClements et al. 2000). Since this field drives the electrophoretic motion, the results will be reduced mobility. On the other hand, the decrease in density associated with the open structure of emulsifier will tend to increase the mobility, since the inertial effect is smaller (Liu et al. 2016b). The most common physical phenomena indicating instability of dispersions is the evolution of droplet size leading to change in droplet size distribution.

For the pectin stabilized emulsion, droplet size increased about 100 nm and 600 nm after heating and freeze thawing, respectively, confirming that coalescence took place after freeze-thaw. This result demonstrated that the interfacial membrane formed by pectin was disturbed by ice crystals more significantly than heat treatment. However, both heat-treated and freeze-thawed emulsions were stable against gravitational separation at all parameters (sonication time, pectin concentration, NaCl concentration and pH condition) considered for evaluation. This might be attributed to the fact that the polysaccharide could enhance the viscosity of emulsion and the relocation rate of droplets can be inhibited (Matos et al. 2015; Fathi et al. 2014). Nevertheless, ζ- potential of pectin emulsions changed significantly ($p<0.05$) at lower pH values in both heating and freeze-thaw treatment indicating that polysaccharides could adsorb to the oil-water interface and form thick interfacial film. However, thermal treatment affected less profoundly on droplet size of emulsions at all pH values compared to freeze-thawing. This observation was attributed to the fact that pectin could provide better protection against partial coalescence because of the comparatively thicker interfacial layer. Homogeneity of the dispersed oil droplets in emulsion was greatly affected by thermal treatment than freeze thawing. This could be ascribed to partial coalescence during heating and complete or near-complete coalescence in freeze thawing treatment (Liu et al. 2016b; Degner et al. 2014). Similarly, increase in droplet size with increase in sodium chloride is due to charge shielding by increase in ionic strength.

4.1.3.4 Rheological properties

In polysaccharide stabilized emulsions, viscosity of the final emulsion formed will be influenced by size distribution, size of the oil droplets, concentration of carbohydrates in continuous phase, thickness of the hydrated interfacial layer, and the steric or electrostatic interactions between the hydrated carbohydrate molecules (Tadros 2015; Matos et al. 2015). In addition to polysaccharide solution characteristics that establishes continuous phase properties, there are other aspects that affect emulsion viscosity such as the rheology of component phases and dispersed-phase volume fraction. However, this fact was mainly observed in highly concentrated emulsion, with the oil phase being above 20% (Pons et al. 1995; Matos et al. 2015). Rheological properties of an emulsion play a significant role in shaping an encapsulation system and optimizing the process conditions such as mixing and pumping.

Pectin is used as an interfacial stabilizer which enhances the viscosity of the continuous phase in flaxseed oil-in-water emulsion. Depending on the emulsion composition, they showed different rheological properties such as Non-Newtonian (Bingham plastics, shear thickening and shear thinning) and Newtonian behavior (Ideal fluid) (Tadros 2015). All the emulsions showed non-Newtonian, shear thickening behavior as viscosity increased with increase in shear rate. In present study, shear thickening behavior was observed and it might attributed to low dispersed phase volume. The decreased consistency index suggested the viscosity of the emulsion system depends on droplet concentration under ultrasonication emulsification. The flow behavior index which is a measure of the extent of shear thickening behavior increased by increase in pH, or sonication time or reduced pectin concentration indicating reduced viscosity in the emulsion. Under ultrasonication assisted emulsification, the increased viscosity affects oil droplet size of emulsion in different ways. Initially, oil droplet movement in the continuous phase is reduced; this hinders sedimentation or creaming leading to long term stability of emulsion. However this does not impact the droplet size at the initial stages. The reduced droplet movement also decreases droplet collisions, leading to stability against coalescence (Silva et al. 2015). Nevertheless, the polyelectrolytic nature of pectin influences its rheological properties. At neutral pH, pectin in solution exhibits a random-coil conformation because of the electrostatic repulsion between the charged groups. Conversely at low pH, pectin in solution changes its rheological property drastically and behaves differently from random coil behavior. Furthermore, when the ionic strength of the solution increases, the electrostatic repulsion is suppressed (Allwyn 2012; Sato et al. 2008).

During emulsification process, the ability of the emulsion to resist re-coalescence process improves the short-term stability of emulsions. The enhanced short-term stability results in smaller droplets for a fixed emulsion component and their concentrations in final formulation. On the other hand, an increased continuous phase viscosity due to pectin action can also influence the instant droplet breakdown during emulsification. It is more important in ultrasonication-assisted emulsification as it helps in decreasing droplet size (Tadros 2015). The transmitted energy from the sonicator is mainly dependent on the viscosity of continuous phase. In other terms it is strongly dependent on viscosity ratio of dispersed phase and continuous phase (Silva et al. 2015).

4.1.3.5 Oxidative stability

A major cause of the acceleration of lipid oxidation in foods is formation of free radicals resulting from the reaction of iron with lipid peroxides that are known to exist in unsaturated lipids. In oil-in-water emulsions, aqueous transition metals are likely to interact with lipid peroxides in the interfacial region that exists between the dispersed oil droplets and the continuous aqueous phase (Mei et al. 1998b). Free radicals arising from the decomposition of peroxides are known to accelerate lipid oxidation; however, little attention has been paid to the prooxidative potential of peroxides originating from emulsifiers at the interface.

Few studies have dealt with the lipid oxidation in polysaccharide stabilized oil-in-water emulsion. Oxidation process in oil droplets in the flaxseed oil-in-water emulsion model results in the development of primary oxidation products such as peroxides. Meanwhile, lipid oxidation process in the emulsion continues to form secondary products by converting low molecular-weight organic acids and polyunsaturated fatty acids (McClements et al. 2000). Lipid hydroperoxides are chosen to measure the extent of lipid oxidation at the early stages of oxidation process. Thiobarbituric reaction substances (TBARS) are considered as secondary oxidation products. In general, storage of flaxseed oil-in-water emulsions at 55 °C led to increase in lipid oxidation rate. This signifies the generation of primary oxidation products (hydroperoxides, free fatty acids, dienes) or secondary oxidation products (trienes, aldehydes, carbonyls) (Scheffler et al. 2009) is greatly temperature dependent. This increased rate of formation of oxidation products is due to the fact that the reduced viscosity of emulsion at high temperature adds the mobility inside the oil droplets. At higher temperature reduced viscosity exposes the internal lipid core to the oxygen present in continuous phase resulting in generation of oxidation products at the interface. However, at higher temperatures the interface of the emulsion also becomes more porous and allows the water soluble pro-oxidants inside the oil droplets resulting in higher rate of oxidation (McClements et al. 2000).

The response of sodium chloride on the emulsified flaxseed oil oxidation may also be elucidated from difference in oil-prooxidants interactions. At highest sodium chloride concentration (120 mM) lipid oxidation was over five times greater than in its absence. Therefore, sodium chloride induced oxidation could be due to increased oil-hydrophilic prooxidants interactions possibly through the ability of salt to increase the charge around

the oil droplet (Mei et al. 1998b). Electric charge on oil droplet in emulsions can be a significant factor in lipid oxidation studies. Many research studies suggests that oil droplet with negative charge (ζ - potential is negative) are more susceptible to oxidation reactions (McClements et al. 2000; Mei et al. 1999). In pectin stabilized emulsions, the charge around the oil droplets is negative due to anionic carboxylic group ($^{-}$COOH) at position C-5 of pectin pyranose ring (Ngouemazong et al. 2015; Thakur et al. 1997). The charge density increased as the pH of the emulsion approaches to neutral or at higher sodium chloride concentration or pectin concentration and influenced the rate of oxidation in emulsion. This is attributed to higher rate of protonation near pH 7.0 and salt induced charge around the droplets. However, in later stages of oxidation reaction, the presence of free fatty acids in emulsified oil also increases the lipid oxidation rate, which could be associated to increased net negative charge around the droplet and their location since they settle at the oil-water interface. Hence, in multiphase pectin-stabilized emulsion systems, pH has the negative impact on lipid oxidation and is dependent on modulation of the interactions between the reactive species at the interface, which in turn depends on the composition of emulsion.

4.2 Structured Pectin, Chitosan Bi-Layer Emulsion: Protection of Flaxseed Oil against Lipid Oxidation

4.2.2 Results

4.2.2.1 Role of chitosan concentration on emulsion characteristics

***Influence on mean droplet diameter and ζ-potential*:** The droplet mean diameter of primary emulsion (zero chitosan concentration) and secondary emulsion as a function of chitosan concentration is depicted in Fig. 4.7a. It was observed that, addition of chitosan to pectin coated primary emulsion significantly increased the droplet size from 709.8 ± 26.35 to 1259.1 ± 47.26 nm. Surprisingly, at chitosan concentration of 4.0 mg/mL, droplet size was reduced and in fact it was not statistically significant ($p>0.05$).

The droplet ζ-potential changed from negative (-18.4 ± 0.68 mV) to positive (+18.7 ± 1.32 mV) with increasing chitosan concentration, which indicated that cationic polysaccharides adsorbed to the anionic pectin coated lipid droplets (Fig. 4.7b). At relatively low concentration of polysaccharide, chitosan showed higher binding affinity for pectin coated droplets with initial steep increase in ζ- potential of the emulsion. This type of ζ-potential-chitosan concentration profile of the emulsion suggests chitosan adsorption at the interface.

***Influence on rheology*:** Rheological properties such as consistency index and flow behavior index of primary emulsion and bi-layer emulsion (as a function of chitosan concentration) are tabulated in Table 4.6. Rheological behavior of emulsions may change depending on concentration of emulsifier at the interface. Addition of chitosan (0.5 mg/mL) layer to pectin-stabilized emulsions reduced the consistency index from 0.253 ± 0.020 to 0.056 ± 0.008 $mPa.s^n$ ($p<0.05$). It clearly suggests that number of droplets (droplet concentration) has the interference in modulating consistency of emulsions. At chitosan concentration 3.0 and 4.0 mg/mL, consistency of secondary emulsion significantly increased (Table 4.6). This increased consistency index was attributed to change in emulsion behavior from shear thickening to shear thinning (Fig. 4.8b). Such fluid matrices are profoundly used in encapsulation of bioactive compounds (Appelqvist et al. 2016).

Flow behavior index of primary emulsion and secondary emulsion behaved uniquely. At chitosan concentration of 1.0 mg/mL, flow behavior index was 0.820 ± 0.042 and in fact it was significantly different from primary emulsion and not significant from secondary emulsion formed with chitosan concentration 4.0 mg/mL. Shift in emulsion

flow behavior from shear thickening to shear thinning can be clearly noticed in Fig. 4.8b represented by shear rate versus viscosity.

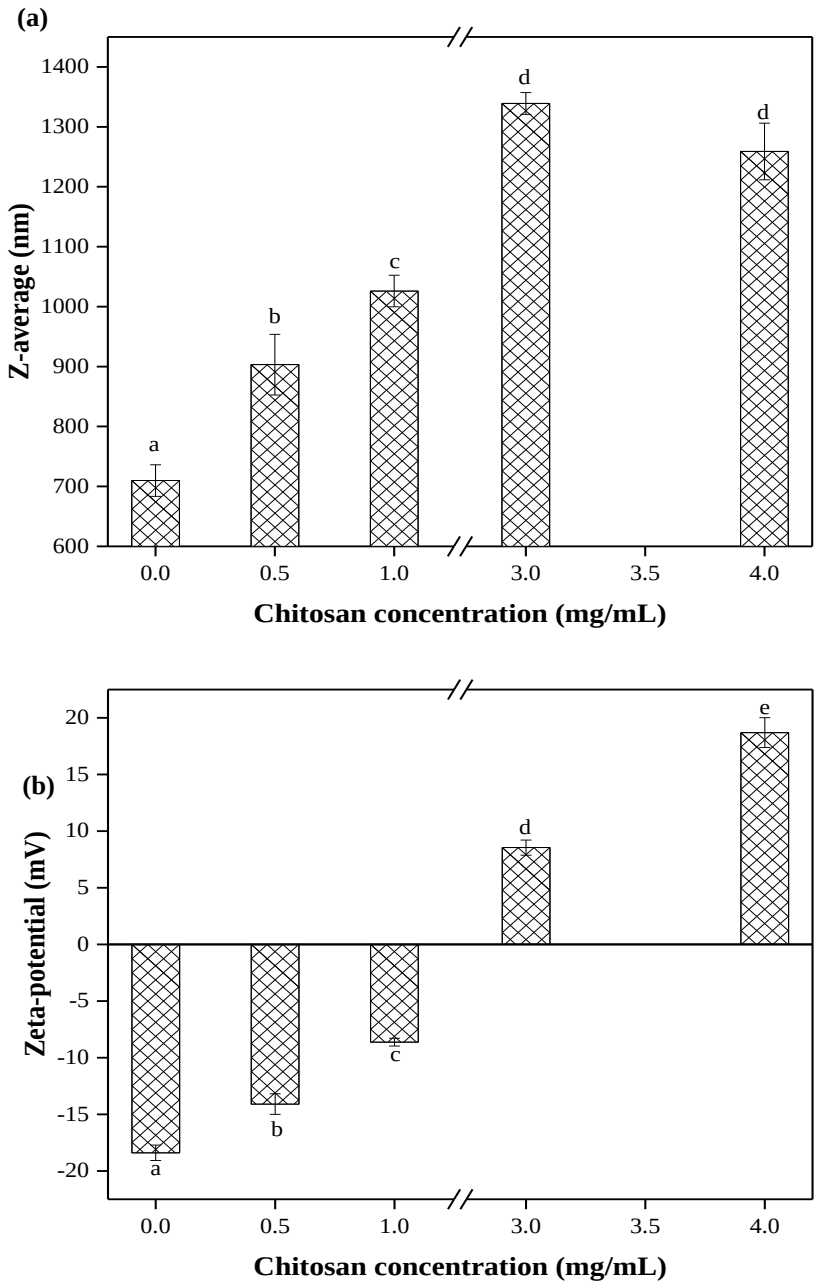


Figure 4.7: Influence of chitosan concentration on (a) droplet mean diameter and (b) ζ-potential of pectin coated emulsion.

Note: Data expressed as mean ± SD (n=3). Significant differences in each column bar are denoted with different letters ($p<0.05$). Chitosan concentration zero referred as pectin-stabilized primary emulsion.

Table 4.6: Influence of chitosan concentration on rheological properties of pectin-stabilized emulsion.

Chitosan Concentration(mg/mL)	Consistency Index, K (mPa.s^n)	Flow Behavior Index, n	Coefficient of Determination (R^2)
0.0	0.253 ± 0.020^{b}	1.502 ± 0.171^{bc}	0.9928
0.5	0.056 ± 0.008^{a}	1.756 ± 0.130^{c}	0.9943
1.0	0.040 ± 0.007^{a}	0.820 ± 0.042^{a}	0.9688
3.0	0.309 ± 0.059^{b}	0.446 ± 0.105^{b}	0.9916
4.0	0.438 ± 0.100^{c}	0.647 ± 0.040^{a}	0.9971

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$). Chitosan concentration zero referred as pectin-stabilized primary emulsion.

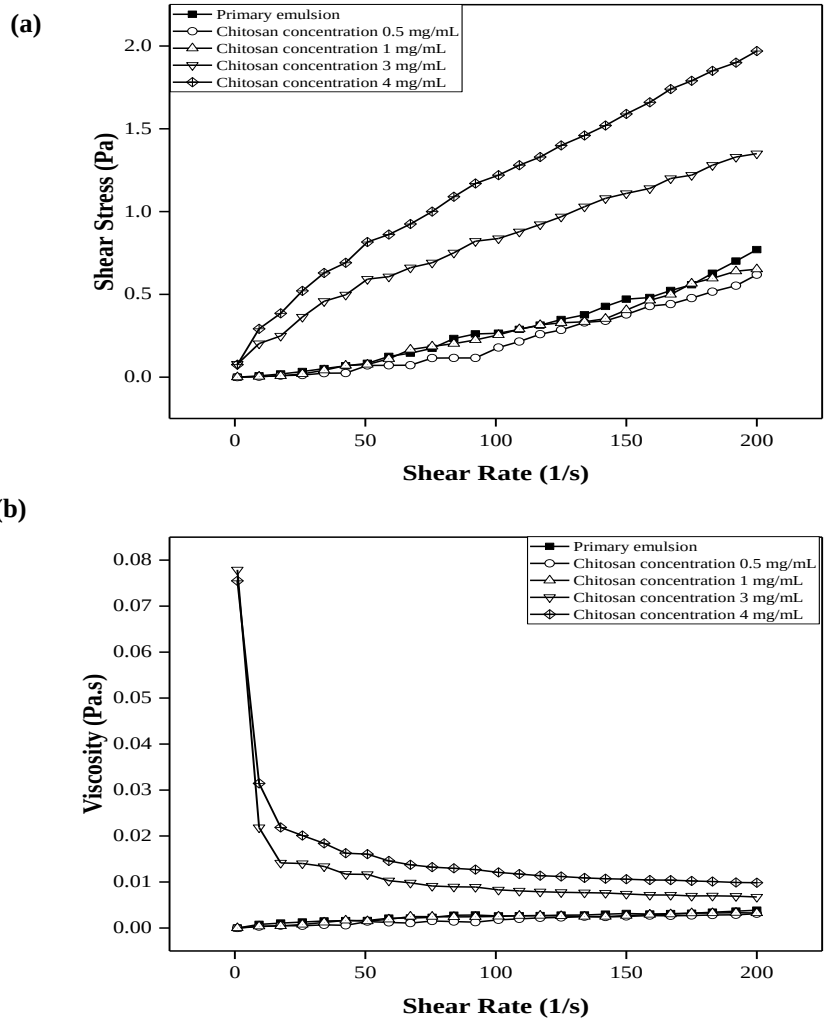


Figure 4.8: (a) Shear stress and (b) viscosity of pectin-stabilized primary emulsion and secondary emulsion (as a function of chitosan concentration).

Note: Primary emulsion refers to emulsion stabilized by pectin at 5.0 mg/mL concentration.

4.2.2.2 Association of environmental stress factors on emulsion properties

***Influence of pH*:** The mean droplet diameter and ζ-potential of the emulsions as a function of pH was measured to provide information about droplet aggregation. In the absence of chitosan, there was significant increase in mean droplet diameter (989.3 ± 23.68 nm) at pH 3.5 (Table 4.7), which can be attributed to extensive droplet flocculation associated with the low net charge on the droplets such as attractive interactions (mainly van der Waals) outweighed the repulsive interactions (mainly steric and electrostatic) (Aoki et al. 2005). In the presence of chitosan, emulsion stability to aggregation depended on the solution pH, which is relative to the critical pH for chitosan to adsorption (Guzey et al. 2006). At pH 3.5 and 5.0, the mean droplet diameter of the emulsion containing chitosan was 1385.4 ± 41.87 and 869.3 ± 38.32 nm, which was significantly different from primary emulsions (Fig. 4.9). The ζ-potential of the secondary emulsion tended to decrease from 27.6 ± 2.34 to 11.7 ± 0.57 mV, suggesting chitosan adsorption to the pectin coated emulsion.

The difference in droplet size at different pH conditions was attributed to the fact that the aggregation of the chitosan-coated droplets began at a fairly similar pH where pectin adsorption began . Consequently, there may have been some aggregation in pectin stabilized droplets before they could be completely covered with chitosan. This might be possible due to charge neutralization or bridging flocculation (Liu et al. 2016a). The pH dependence of chitosan adsorption to the pectin coated oil droplet surface influence the affinity of emulsion to droplet aggregation as a function of pH.

Table 4.7: Role of pH on mean droplet diameter and ζ-potential of primary and secondary emulsion.

Sample Name	pH	ζ-potential (mV)	Z-average (nm)
Primary emulsion	3.5	-14.4 ± 0.87^{b}	989.3 ± 23.68^{c}
	5.0	-15.6 ± 1.25^{a}	648.2 ± 33.75^{a}
Chitosan concentration 4.0 mg/mL	3.5	27.6 ± 2.34^{c}	1385.4 ± 41.87^{d}
	5.0	11.7 ± 0.57^{d}	869.3 ± 38.32^{b}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$). Primary emulsion refers to emulsion stabilized by pectin at 5.0 mg/mL concentration.

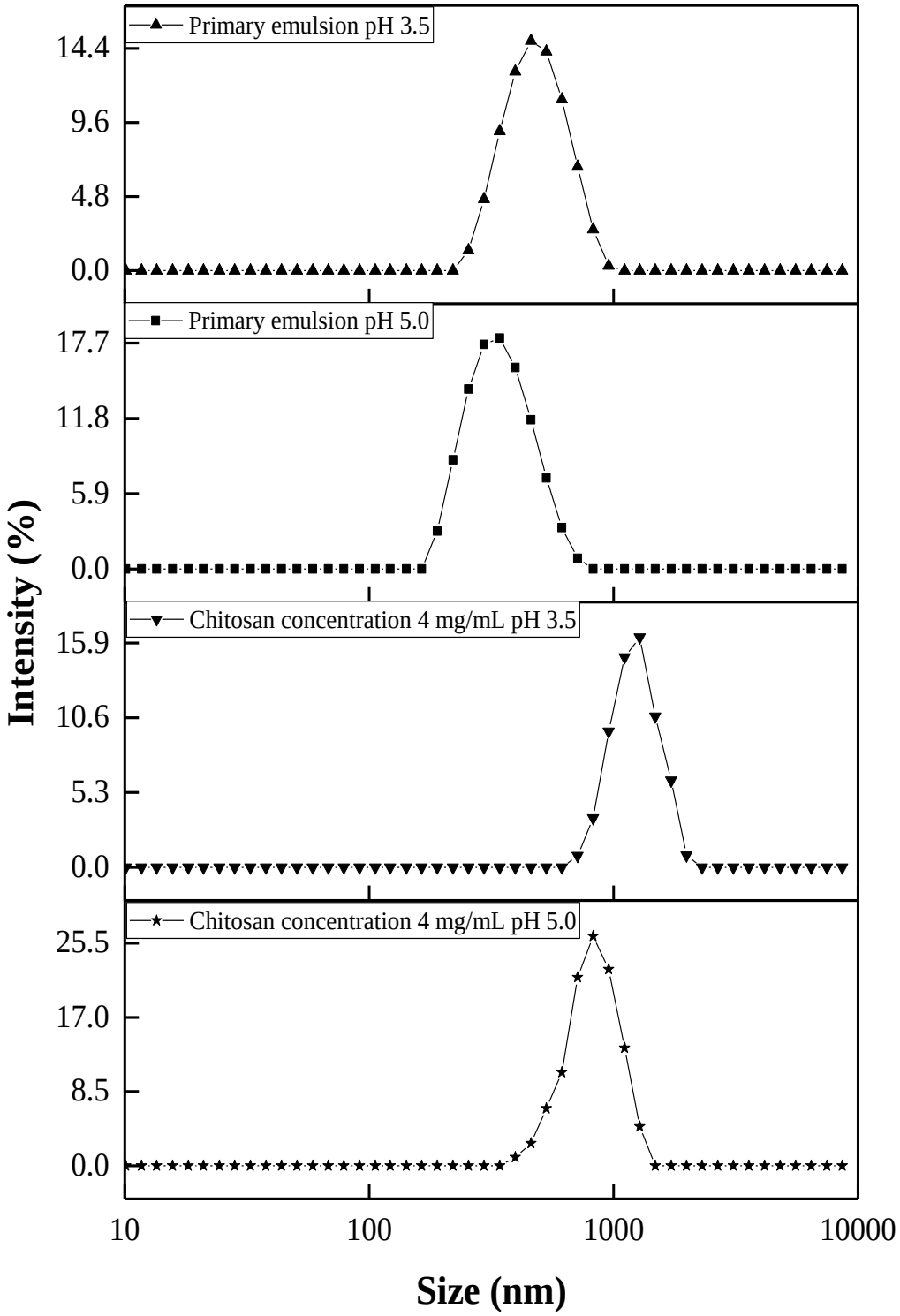


Figure 4.9: Droplet size distribution curves of primary and secondary emulsion at varied pH conditions.

Note: Primary emulsion refers to emulsion stabilized by pectin at 5.0 mg/mL concentration.

***Influence of NaCl*:** Addition of salt may destabilize the emulsion droplets. Therefore, experiments were performed to study the influence of sodium chloride on mean droplet diameter and ζ-potential of primary emulsion and secondary emulsion was measured (Table 4.8). Absolute value of charge in primary emulsion increased from -18.0 ± 1.23 mV to -21.6 ± 0.69 mV ($p<0.05$). In contrast to this, in bi-layer emulsion, ζ-potential value changed from 11.7 ± 1.55 mV to -6.1 ± 0.43 mV. In low concentration of sodium chloride, secondary emulsion droplet charge remained positive. These results might be ascribed to accumulation of anionic (Cl^-) or cationic (Na^+) ions at the interface. In addition electrostatic screening effects of sodium chloride could decrease the absolute value of emulsion droplet charge and, under high salt concentration, it might even lead to charge reversal (Klinkesorn et al. 2009).

Droplet sizes of pectin coated primary emulsion increased with the addition of sodium chloride ($p<0.05$). Similar trend was observed in secondary emulsions; however, at sodium chloride concentration ≤ 50 mM, droplet size increment was not significant (Table 4.8) (Fig. 4.10). Increase in droplet size of secondary emulsion at 80 mM sodium chloride might be attributed to droplet aggregation in chitosan coated droplets. This might be due to charge neutralization or bridging flocculation. In addition, decreased absolute ζ-potential around the oil droplet was also responsible for increase in droplet size at higher salt concentration. However, droplet instabilities such as flocculation and droplet bridging can occur at low absolute value of charge during storage which can be overcome through steric stabilization in polysaccharide stabilized emulsions.

Table 4.8: Role of NaCl on mean droplet diameter and ζ-potential of primary and secondary emulsion.

Sample Name	NaCl concentration (mM)	ζ-potential (mV)	Z-average (nm)
Primary emulsion	30	-18.0 ± 1.23^{b}	977.6 ± 17.68^{a}
	50	-20.9 ± 0.69^{ab}	1155.1 ± 48.35^{b}
	80	-21.6 ± 1.73^{a}	1395.8 ± 53.31^{c}
Chitosan concentration 4.0 mg/mL	30	11.7 ± 1.55^{e}	1536.7 ± 18.84^{d}
	50	7.6 ± 0.27^{d}	1609.2 ± 28.65^{d}
	80	-06.1 ± 0.43^{c}	1948.3 ± 67.45^{e}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$). Primary emulsion refers to emulsion stabilized by pectin at 5.0 mg/mL concentration.

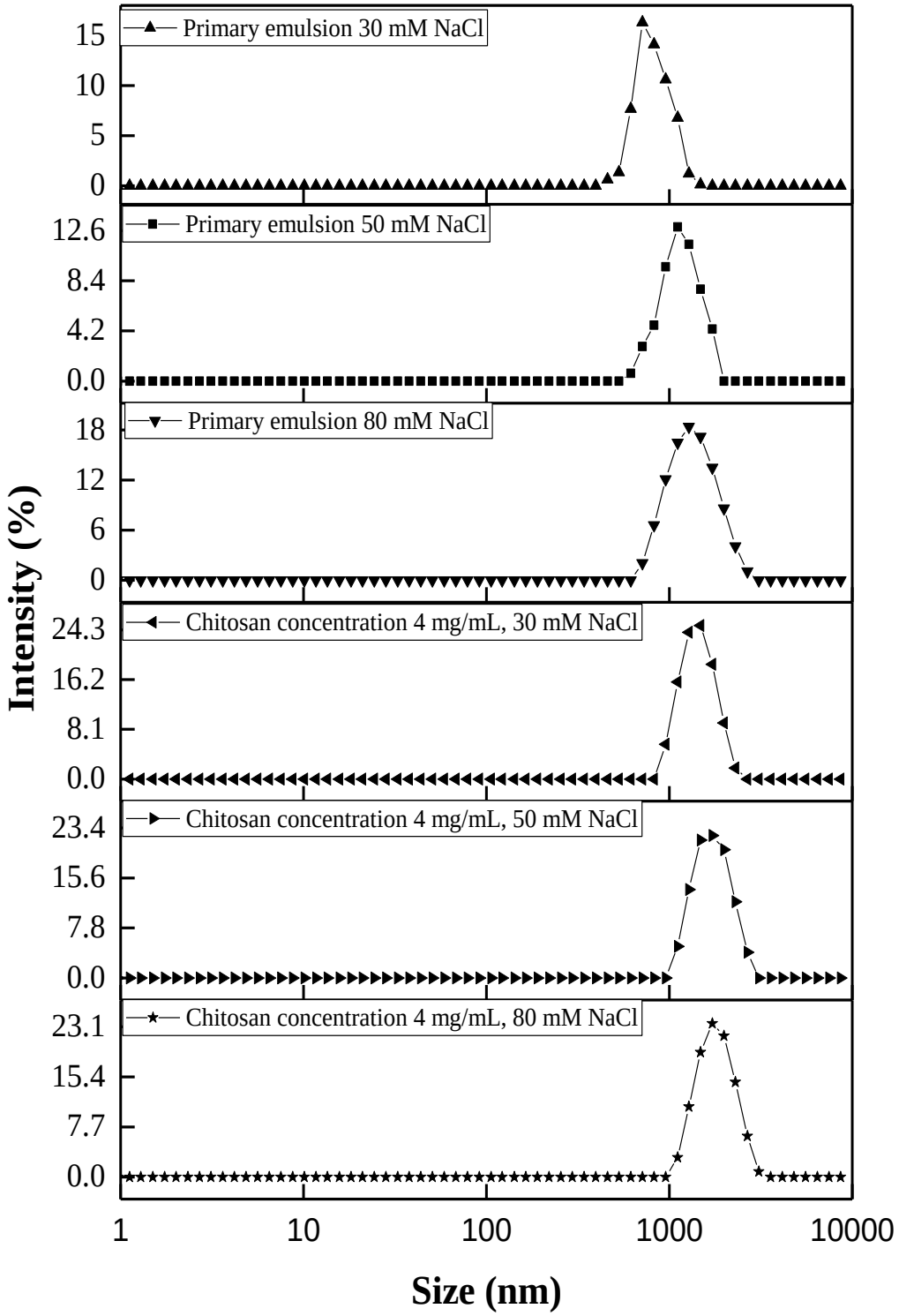


Figure 4.10: Droplet size distribution curves of primary and secondary emulsion at varied NaCl concentration.

Note: Primary emulsion refers to emulsion stabilized by pectin at 5.0 mg/mL concentration.

***Influence of thermal treatment*:** Thermal treatment may destabilize the emulsion matrix, therefore thermal treatment was carried out and change in droplet size and ζ-potential was measured. Primary emulsion stabilized with pectin was affected by thermal treatment. This was supported by change in mean droplet diameter (Fig. 4.11a). At 40 ºC droplet size was 1025.1 ± 74.50 nm, and it increased significantly to 3388.4 ± 300.65 nm at 100 ºC. In the presence of chitosan, secondary emulsion demonstrated stability against thermal destabilization. However, at higher temperatures (≥ 90 ºC) secondary emulsion stabilized by chitosan at concentration 3.0 and 4.0 mg/mL formed gel network during storage for 24 hrs. At low chitosan concentration (0.5 mg/mL), droplet aggregation in primary emulsion was inhibited even at 100 ºC. Increased concentration to the interface of primary layer resulted in increased droplet diameter with respect to temperature increase caused due to droplet bridging and flocculation.

In the absence of chitosan in primary emulsion, absolute value of charge around the droplet increased significantly with respect to increase in temperature (Fig. 4.11b). At chitosan concentration 0.5 mg/mL, the negative absolute value of charge increased from -14.8 ± 0.68 mV to -17.6 ± 1.02 mV, suggesting that chitosan did not cover pectin coated oil droplet completely. Surprisingly, at chitosan concentration 1.0 mg/mL, ζ-potential change has not significant. Conversely, at chitosan concentration 3.0 and 4.0 mg/mL absolute value of ζ-potential increased with increase in temperature, however at ≥ 90 ºC, gelling occurred due to droplet coalescence and reduced droplet repulsion at higher temperature.

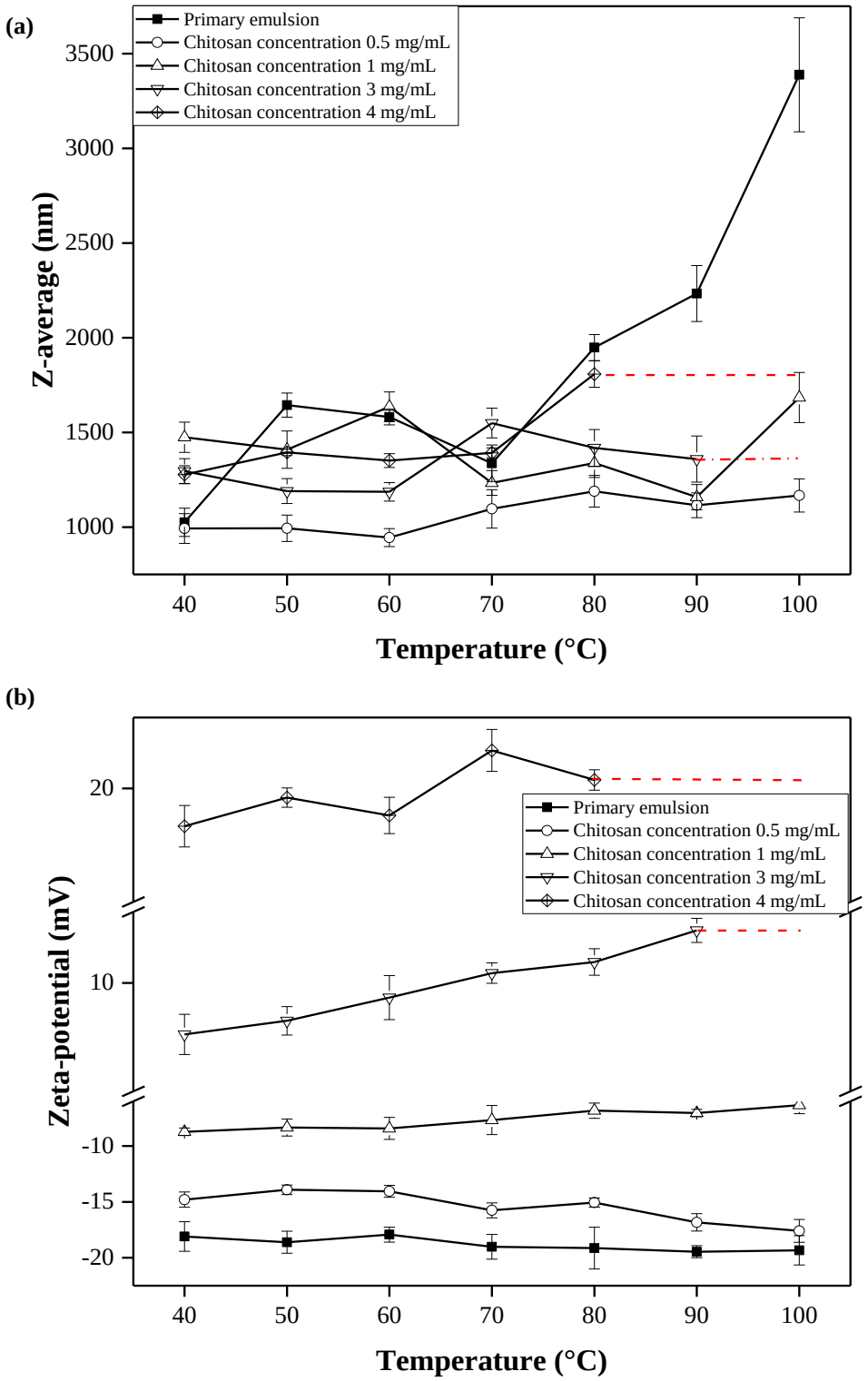


Figure 4.11: Effect of thermal treatment on (a) mean droplet diameter and (b) ζ-potential of pectin-stabilized primary emulsion and secondary emulsion (as a function of chitosan concentration).

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$). Primary emulsion refers to emulsion stabilized by pectin at 5.0 mg/mL concentration.

4.2.2.3 Stability of structured bi-layered emulsion

***Influence of chitosan concentration*:** In the presence of chitosan, the emulsion stability to droplet flocculation was strongly dependent on the chitosan concentration (Table 4.9a). In the presence of high amounts of chitosan, emulsions showed good stability to droplet flocculation, but there was an increase in the droplet size at pH 5.0 (Table 4.9b). In pectin-stabilized primary emulsion, the droplet growth ratio varied from 0.101 ± 0.005 to 0.498 ± 0.012 ($p<0.05$).

***Influence of pH*:** At pH 5.0, the instability of pectin and chitosan coated emulsions could be due to less electrostatic interactions presented between negative patches of pectin molecules and positive chitosan at this pH (Table 4.9b). The growth of the oil droplets occurred at all pH conditions. At pH 5.0, the droplet growth could be due to destabilized pectin emulsion over time with increasing conformational and interaction changes mentioned above. In addition, chitosan concentration was too low to completely saturate pectin-coated oil droplets at pH 5.0 due to degree of saturation variation with pH condition. The charge density of chitosan emulsion showed a significant impact on the emulsion stability.

Table 4.9: Influence of (a) chitosan concentration (b) pH of emulsion (c) NaCl concentration on droplet growth ratio at 40 ºC

(a)

Sample Name	Chitosan concentration (mg/mL)	Droplet Growth Ratio at 40 ºC
Primary emulsion	0.0	0.435 ± 0.024^{b}
Bi-layer emulsion	0.5	0.498 ± 0.012^{c}
	1.0	0.400 ± 0.018^{b}
	3.0	0.111 ± 0.015^{a}
	4.0	0.101 ± 0.005^{a}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$). Primary emulsion refers to emulsion stabilized by pectin at 5.0 mg/mL concentration.

(b)

Sample Name	pH	Droplet Growth Ratio at 40 ºC
Primary emulsion	3.5	0.364 ± 0.008^{c}
	5.0	0.491 ± 0.010^{d}
Chitosan concentration 4.0 mg/mL	3.5	0.214 ± 0.009^{b}
	5.0	0.180 ± 0.006^{a}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$). Primary emulsion refers to emulsion stabilized by pectin at 5.0 mg/mL concentration.

(c)

Sample Name	NaCl concentration (mM)	Droplet Growth Ratio at 40 ºC
Primary emulsion	30	0.275 ± 0.024^{b}
	50	0.195 ± 0.019^{a}
	80	0.268 ± 0.021^{b}
Chitosan concentration 4.0 mg/mL	30	0.312 ± 0.027^{bc}
	50	0.341 ± 0.022^{c}
	80	0.303 ± 0.018^{bc}

Note: Data expressed as mean ± SD (n=3). Significant differences in each column are denoted with different letters ($p<0.05$). Primary emulsion refers to emulsion stabilized by pectin at 5.0 mg/mL concentration.

***Influence of NaCl*:** In any emulsion, stability is defined by electrostatic interactions between droplets and steric mechanism, particularly in polysaccharide stabilized emulsions. In our current studies, sodium chloride reversed the charge of droplet from positive to negative (Table 4.9c). However, phase separation was not observed in any emulsion prepared at different salt concentrations. This clearly suggests that steric stabilization overweighed destabilization by net charge neutralization and flocculation. The droplet growth ratio was varied from 0.195 ± 0.019 to 0.268 ± 0.021 in primary emulsion and 0.303 ± 0.018 to 0.341 ± 0.022 ($p<0.05$) (Table 4.9c).

4.2.2.4 Lipid oxidation in bi-layer emulsion matrix

Emulsion storage and composition promote gradual lipid oxidation with decreases in shelf life stability due to continuing chemical reactions set up by the initial peroxidations. Chitosan at a concentration of 4.0 mg/mL decreased the peroxidation in emulsion samples significantly (Fig. 4.12a). Lipid oxidation in emulsion samples in the absence of chitosan were higher compared to the presence of all chitosan concentration. Developments of lipid peroxides in chitosan concentration of ≥ 1.0 mg/mL during storage above four days were lower compared to emulsion containing low chitosan concentration. TBARS development during storage reflects the stability of bi-layered emulsion against oxidation. Fig. 4.13 illustrates influence of pH condition on lipid oxidation in structured bi-layer emulsion. This experiment showed that variations in emulsion pH condition have a major impact on oxidation, however, lipid oxidation hindered in by bi-layer emulsion. This lipid oxidation inhibition was possibly due to high net positive charge around the oil droplets.

Sodium chloride containing primary emulsion that contained more than 20 mM showed low level of lipid oxidation. Similar results were observed in bi-layer emulsion (Fig. 4.14a). The results indicate that the order of lipid oxidation inhibition activity of sodium chloride in bi-layer emulsion was 80 mM ≤ 50 mM ≤ 30 mM. The TBARS values of primary and bi-layer emulsion are shown in Fig. 4.14b. It was confirmed that, during initial storage days (Day 4) development of secondary lipid oxidation products were high in primary emulsion containing 80 mM. This might be due to charge screening effect of sodium chloride at higher concentration (Klinkesorn et al. 2009). However, after initiation of emulsion oxidation, sodium chloride inhibited the development of secondary oxidation products in emulsion.

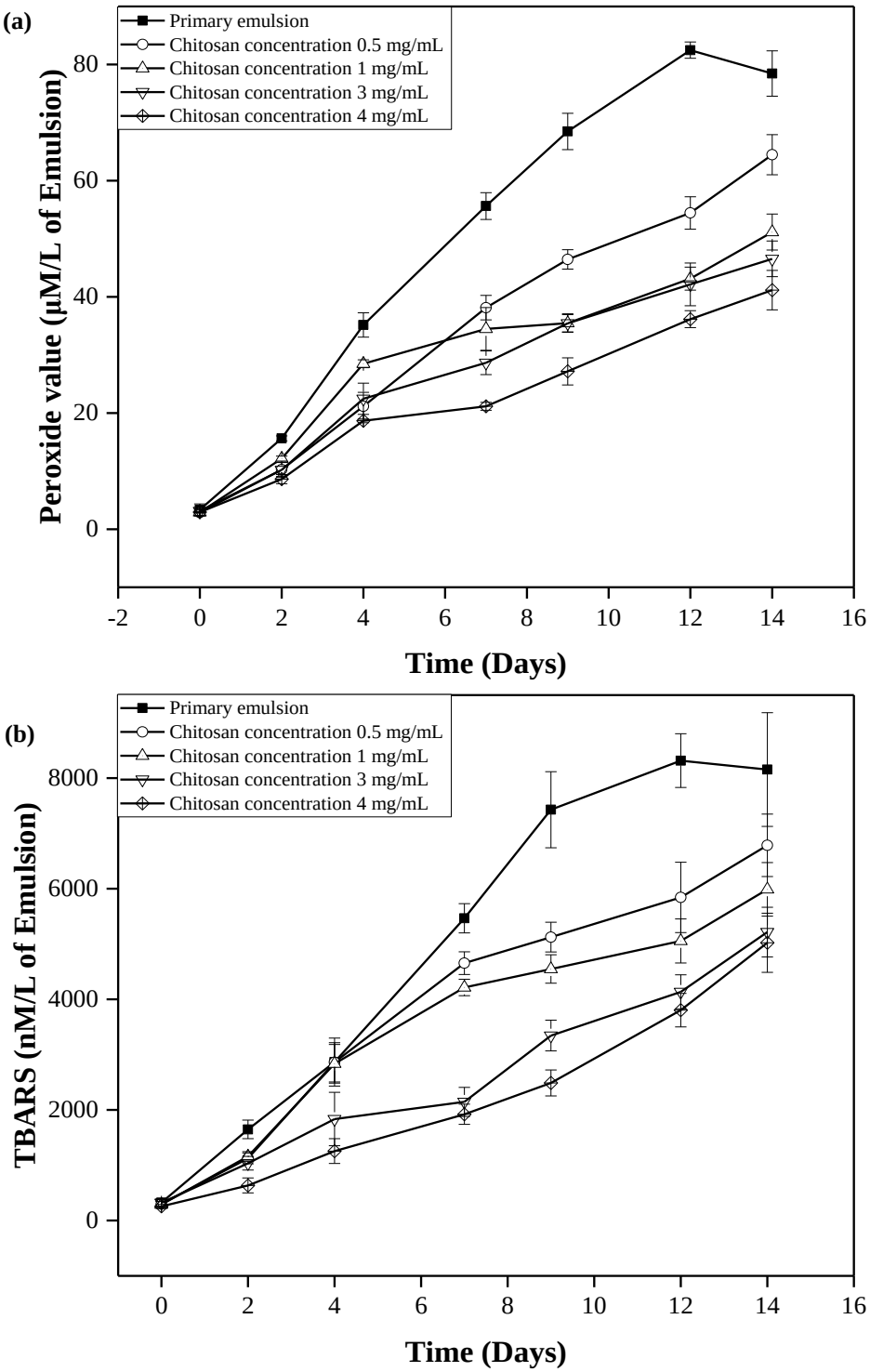


Figure 4.12: Influence of chitosan concentration on (a) peroxide value and (b) TBARS of pectin-stabilized flaxseed oil-in-water emulsion at 40 ºC.

Note: Characterization of lipid oxidation on day zero of storage in Figure 4.12 refers to measurement after 60 min of sonication. Data expressed as mean ± SD (n=3).

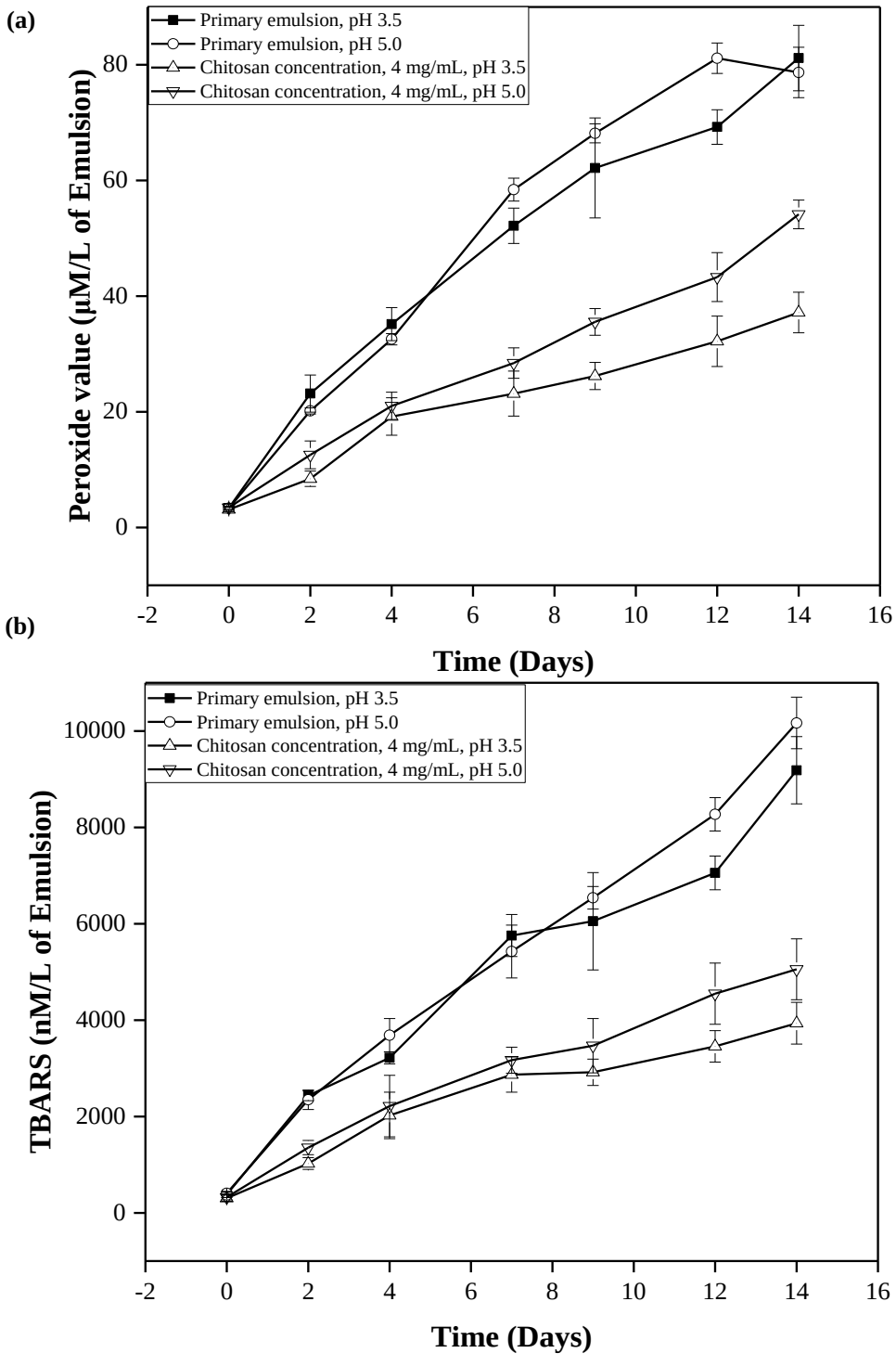


Figure 4.13: Influence of emulsion pH on (a) peroxide value and (b) TBARS of pectin-stabilized flaxseed oil-in-water emulsion at 40 ºC.

Note: Characterization of lipid oxidation on day zero of storage in Figure 4.13 refers to measurement after 60 min of sonication. Data expressed as mean ± SD (n=3).

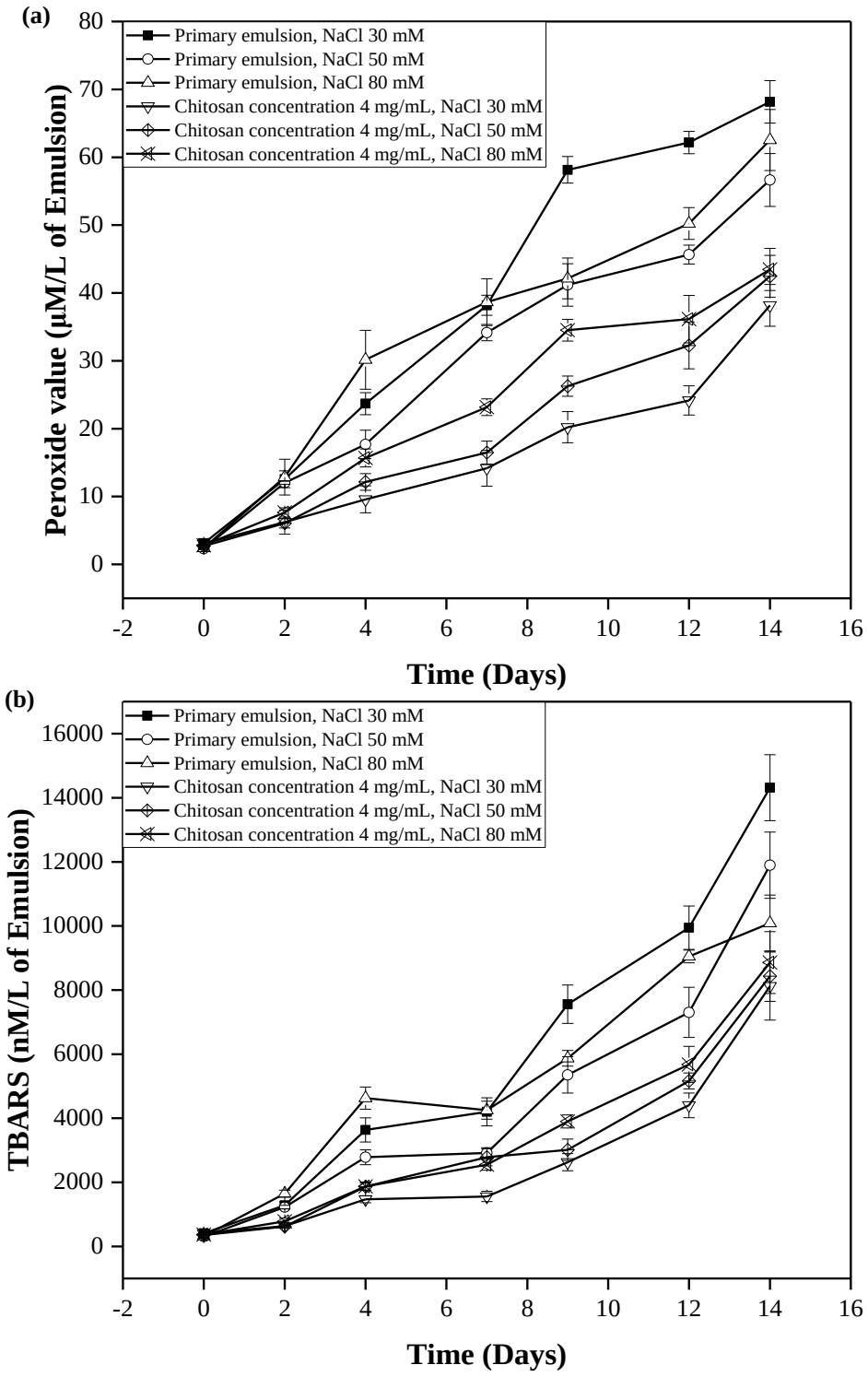


Figure 4.14: Influence of NaCl concentration on (a) peroxide value and (b) TBARS of pectin-stabilized flaxseed oil-in-water emulsion at 40 ºC.

Note: Characterization of lipid oxidation on day zero of storage in Figure 4.14 refers to measurement after 60 min of sonication. Data expressed as mean ± SD (n=3).

4.2.3 Discussion

4.2.3.1 Emulsion characteristics as a function of chitosan layer

Overall, the ζ-potential of the emulsion went from negative at low chitosan concentration to positive at high chitosan concentration. This kind of ζ-potential versus chitosan concentration profile is typical for bi-layer emulsion formed using oppositely charged polysaccharides (Guzey et al. 2006; Liu et al. 2016a). The mean droplet diameter remained high (d > 700 nm) from chitosan concentration 0.5 to 1.0 mg/mL, but there was an appreciable increase in its value at chitosan concentration 3.0 and 4.0 mg/mL. The droplet size distribution indicated that this was due to the formation of a population of relatively large droplets at the higher chitosan concentration. Results indicated that appreciable droplet aggregation occurred in the emulsions at chitosan concentration around near zero ζ-potential, which can be attributed to the relatively low net droplet charge and therefore relatively weak electrostatic repulsion between the droplets (McClements 2010; Guzey et al. 2006; Aoki et al. 2005). It was observed that the ζ-potential on the pectin-coated lipid droplets actually decreased in absolute value when chitosan was increased; however, it also reversed the charge. Viscosity was larger in the emulsions containing higher amount of chitosan, in fact rheological flow behavior changed from shear thickening to shear thinning. This change in behavior can be attributed to droplet flocculation and supports the hypothesis that flocculation was much less when higher amount of chitosan has added to the interface (Appelqvist et al. 2016; Silva et al. 2015). In addition, as the dispersed phase volume decreases (increase of effective volume fraction) with increase of chitosan concentration, it induced the cationic charge on droplets. Hence, change in rheological behavior might also attribute to increased formation of large aggregates due to bridging of flocculation. Flocculated emulsions may exhibit a high low-shear viscosity and shear thinning behavior, which can be attributed to the entrapment of certain amount of continuous phase within the aggregated structure leading to an increase in effective volume fraction of these entities. Progressive deformation and disruption of the flocs do occur with applying a shear field resulting in lower viscosity at elevated shear rates (Zinoviadou et al. 2012; Wang et al. 2016).

4.2.3.2 Environmental stress factors

Commercial products containing lipid droplets may be subjected to various kinds of thermal treatments during their production, storage and utilization, *e.g.,* pasteurization, sterilization, temperature fluctuations, and cooking (Liu et al. 2016b). It is therefore important to establish the influence of heating on the stability and physicochemical properties of pectin-chitosan stabilized emulsions. In addition to this sodium chloride and pH of emulsion were considered as stress factors. Typically, carboxyl groups ($-CO_2H \leftrightarrow -CO_2^-$) of pectin molecules have p*Ka* ~ *3.3* and the amino group ($–NH_3^+ \leftrightarrow NH_2$) of chitosan molecule have p*Ka* ~ 6.5. Pectin displayed the characteristic behavior of an anionic polyelectrolyte (Moreau et al. 2003). The zeta-potential of pectin stabilized primary emulsions remained negative as the pH was increased which could be attributed to the fact that the pK_a values of pectin is around pH 2.9-3.3. Whilst, pectin layer start lose its negative charge as the pH is lowered near this value. The pH of final emulsion after the addition of chitosan layer on pectin stabilized emulsion was between the range of p*K*a of both pectin and chitosan due to their opposite charges. However, in an acid environment chitosan behaves as polycationic electrolyte and this yields antifungal or antimicrobial activities since cations can bind to anionic sites on bacterial and fungal cell wall surfaces. However, at higher pH, it tends to lose its charge and may precipitate from solution due to deprotonation of the amino groups. It should be also underlined that the p*K*a of the ionizable groups on a polyelectrolyte can be shifted from their values in solution due to their local electrostatic environment. This change in p*K*a can alter the pH where one would expect a polyelectrolyte layer to become desorbed from an oil-water interface (Sperber et al. 2009).

Sodium chloride as stress factors affected the droplet size and zeta-potential uniquely. At low concentration of sodium chloride, measured secondary emulsion droplet charge remained positive. These results might be ascribed to the accumulation of anionic (Cl^-) or cationic (Na^+) ions at the interface. In addition, electrostatic screening effects of sodium chloride could decrease the absolute value of emulsion droplet charge and, under high salt concentration, it might even lead to charge reversal (Klinkesorn et al. 2009). If the pH is adjusted using different anions, the solution viscosity will be different because of the screening of the protonated amino groups or steric hindrance exerted by the anion and the intramolecular electrostatic repulsion. For example carboxylate ions increase the chain

stiffness of chitosan, whereas the chloride ion is too small to affect the chain stiffness (Chang et al. 2015a; Costa et al. 2015). In addition, this effect can be attributed to the fact that zeta-potential of a droplet decreases when the ionic strength of the surrounding aqueous phase increases due to electrostatic screening effects (McClements 2015). The opaque droplet-rich region above the serum phase appeared homogeneous at lower holding temperatures (40 to 80 ºC), but was cracked, shrunken, and gel-like at higher holding temperatures (90 and 100 ºC). This was attributed to extensive droplet flocculation following thermal denaturation of the adsorbed proteins. The formation of a particle-network at the highest temperature probably accounts for the fact that no creaming was observed because the movement of the droplets was retarded (Liu et al. 2016b; Tadros 2015). However, higher the ionic strength, the lower the solubility of chitosan. The addition of an electrolyte will reduce the electrostatic repulsion between the positively charged chitosan chains, and thus results in a salting-out effect leading to the precipitation of chitosan from the solution (Chang et al. 2015a).

A tendency for droplet aggregation to occur in polysaccharide stabilized bi-layer emulsions has previously been attributed to thermal destabilization. At higher temperature gelation was observed in bi-layer emulsions (Mantovani et al. 2016). Gelation is the process of formation of gel from sol. A gel can be recognized as an intermediate state between liquid and solid, that is, they are liquid rich system, yet possesses solid-like properties with no flow under gravity. Structurally, emulsion gels can be of two types: (1) dispersed droplets filled biopolymer gels, and (2) particulate gels. In the former droplets are randomly dispersed in a biopolymer matrix that imparts gelation (Torres et al. 2016). In emulsions, gelation is caused by the network of biopolymer or hydrocolloid molecules in the continuous phase and the presence of droplet in the matrix either strengthen or weaken the gel depending on their interfacial properties. However, strength of gels depends on the nature and extent of inter-droplet interactions and the dispersed phase volume fraction (Montes de Oca-Ávalos et al. 2016).

An inter-droplet interaction involves attractive and repulsive interactions. In the case of attractive emulsion gelation, aggregation is caused by the attraction between the emulsion droplets. If attractive interactions can be induced among the repulsive droplets, secondary attractive minima in their inter-droplet pair potential can be developed which would lead to droplet aggregation resulting in gelation (Torres et al. 2016). However,

depletion attraction and salt induced attraction was also responsible for gelation in emulsions. Due to the osmotic pressure difference between the surrounding continuous phase and the depleted inter-droplet region, a net force is exerted on the droplets to aggregate, which is manifested as the attractive interactions (Soukoulis et al. 2016). In electrostatically stabilized emulsions, the addition of an appropriate amount of ionic salt screens the charge on the droplets, thereby reducing the repulsive barrier between the droplets, leading to droplet aggregation. Repulsive gelation in emulsions happens when volume fraction of ionic emulsifier stabilized droplets with strong short range repulsion increased to an extent that they become packed and the emulsion attains yield stress (Zhao et al. 2016).

4.2.3.3 Stability of structured bi-layered emulsion

In the presence of chitosan, the emulsion stability to droplet flocculation was strongly dependent on the chitosan concentration. In the presence of high amounts of chitosan, the emulsions showed the good stability to droplet flocculation, but there was an increase in the droplet size at pH 5.0. At pH 5.0, the instability of pectin and chitosan coated emulsions could be due to the instability of the pectin emulsion and few electrostatic interactions between negative patches of pectin molecules and positive chitosan at this pH (Fathi et al. 2014; Ogawa et al. 2004). The growth of the oil droplets occurred at all pH conditions. The droplet growth could destabilize pectin emulsion over time with increasing conformational and interaction changes mentioned above. The result of the accelerated droplet growth test largely supports the results from the viscosity and particle size measurements. Nevertheless, there were some differences, which can be attributed to the different sensitivities of the techniques to particle flocculation. Droplet flocculation is relatively limited at low temperatures, but becomes extensive at higher temperatures, leading to decrease in emulsion viscosity and increase in droplet instability (Tadros 2015). In addition, in lower pH condition more than 90% of the free carboxyl groups in pectin are ionized, while at higher pH condition all free carboxyl groups are ionized. From the ionization status, a higher pH would be between for pectin-chitosan interaction but at pH $\geq$ 5.0 pectins are no longer stable and start to degrade. Pectin chain degradation occurs due to β-elimination, whereby the glycosidic bonds of the pectin backbone are cleaved, consequently reducing the molecular weight (Wüstenberg 2014; Sperber et al. 2009).

4.2.3.4 Lipid oxidation in bi-layer emulsion matrix

The observed slow increase in lipid oxidation during storage suggests that cationic charge around the droplets may be useful in further increasing the oxidative stability of the emulsion encapsulation system (Berton-Carabin et al. 2014). Free radicals arising from decomposition of peroxides are known to accelerate lipid oxidation; however, little attention has been paid to the prooxidative oxidation potential of peroxides originating from polysaccharides. Chitosan, noted for its imperviousness to peroxidation, may be able to inhibit lipid oxidation under different conditions (Tomida et al. 2010). Transition metals are well known for their ability to decompose peroxides. Since transition metal peroxide interactions will both decompose peroxides (resulting in a decrease in peroxide concentrations) and produce free radicals which may promote oxidative reactions (resulting in an increase in peroxides), changes in peroxide concentrations in the presence of metals actually represents a balance between peroxide formation and decomposition (Frankel 2014; Berton-Carabin et al. 2014).

In the presence of sodium chloride, the initial decrease in peroxides could represent the ability of sodium chloride to decompose pre-existing peroxides, resulting in initiation of free radical formation which eventually favors peroxide formation during the later stages of oxidation (Osinchak et al. 1992). Lower concentrations of sodium chloride ($\geq$ 50 mM) caused an increase instead of a decrease in peroxide concentration. This could be due to the inability of the lower sodium chloride concentrations to substantially decrease peroxide value while still producing enough free radicals to favor peroxide formation. Negative electric charge around the oil droplets also attracts prooxidation factors resulting in acceleration of lipid oxidation (Frankel 2014; Berton-Carabin et al. 2014).

Chapter 5

Conclusions

In the first part of the study, the influence of sonication time, biopolymer concentration, sodium chloride concentration and emulsion pH on the physical and oxidative stability of oil-in-water emulsion was characterized. The minimum droplet size achievable and the minimum amount of emulsifier-to-oil required to produce small droplets depended on the sonication time. At five minute of sonication time, droplet size value was 517.6 ± 18.25 nm. Although pectin was better at producing small droplets at low emulsifier concentrations during sonication than the higher concentration, it was much more susceptible to droplet aggregation when environmental or solution conditions were altered. Extensive droplet aggregation occurred in the pectin emulsions around their protonation point (pH 6.0-7.0), at high salt concentrations (≥ 40mM NaCl), and at freeze thawing condition, which was attributed to changes in steric and hydrophobic interactions between droplets. This study showed that pectin emulsions with good stability to a range of environmental stresses. The information generated in this study will be useful for the rational selection of biopolymer for use in encapsulation.

In bi-layer emulsion studies, peroxides originating from pectin-chitosan seem to be able to participate in oxidative reactions, especially in the presence of sodium chloride and at pH condition 5.0. Strong electrostatic interactions between pectin primary layer over flaxseed oil droplets and interface can be balanced effectively by fabricating another layer using oppositely charged chitosan. This allows regulation of complexation process and in turn regulation of encapsulated flaxseed oil. Pectin-chitosan stabilized bi-layer flaxseed oil-in-water emulsions with high oxidative and physical stability can be prepared at pH 4.0. Sodium chloride reversed charge around the bi-layer emulsion. The ability of pectin-chitosan bi-layer at interface to decrease the oxidative deterioration of flaxseed oil could be due to the formation of a cationic emulsion droplet interface, which can repel prooxidative metals. This knowledge is particularly useful for utilization of the emulsions as encapsulation systems for incorporation of functional lipids into foods with relatively high ionic strengths, temperature and pH variation.

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Dissemination

Book Chapters

1. *Lohith Kumar DH, Preetam Sarkar.* **Nanoemulsion as Nutrient Delivery System in Food: Concept-Structure-Properties, In Nanoscience in Food and Agriculture**, Editors: Shivendu Ranjan, Nandita Dasgupta and Eric Lichtfouse. Series-Sustainable Agriculture Reviews-***Springer-Nature Publication*** (Accepted).

2. Lohith Kumar DH, Preetam Sarkar. **Potential of Nanotechnology in Diary Processing-A Review**, **In Sustainable Biological Systems for Agriculture: Emerging Issues in Nanotechnology, Biofertilizers, Wastewater, and Farm Machines**, Editors: Megh R. Goyal. Series- Innovations in Agricultural and Biological Engineering-***Apple Academic Press-CRC Press Publication***-2017, ISBN 9781771886147, (Published).

Refereed Articles

1. **Lohith Kumar DH, Preetam Sarkar.** *Effect of pH on physical and chemical (oxidative) properties of ultrasound-assisted flaxseed oil-in-water emulsion stabilized by an anionic biopolymer,* **Journal of Food Measurement and Characterization**, (**Submitted**).

Conferences

1. *Lohith Kumar DH, Preetam Sarkar.* **Influence of Biopolymer Concentration on Physical and Rheological Properties of Flaxseed Oil-in-water Emulsion**, *XXIV Indian Convention of Food Scientists & Technologists*, 2015.